REACTIVATION OF HEAT INACTIVATED INFLUENZA VIRUS BY RECOMBINATION

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The phenomenon of multiplicity reactivation in bacterial virus infections has been described by Luria (1947), Luria and Dulbecco (1949). When a bacteriophage is treated by ultra-violet irradiation, the plaque count that it will produce under standard conditions is reduced to a certain level. This will correspond to conditions in which there will be no opportunity for more than one phage particle to make contact with any given bacterial cell. If, however, conditions are arranged so that each cell may take up 2 or more phage particles and platings are subsequently made, the plaque count is proportionately higher. It appears that on occasion when 2 "nonviable" virus particles enter a cell simultaneously, they can together initiate virus multiplication although neither alone could do so. The original concept that this was due to the possibility of replacing damaged genes by mutual assistance between 2 or more phage genomes has been discarded without any clear alternative being provided (Dulbecco, 1952).

Working with influenza viruses in the allantoic cavity, Henle and Liu (1951) observed a somewhat analogous phenomenon with virus partially inactivated by ultra-violet light. If undiluted dialysed virus is irradiated so that its infective titre is reduced to 1 p.c. of the original, its behaviour when inoculated undiluted can be compared with 1 : 100 dilution of the original, which has the same nominal infectivity. Henle and Liu found that the time of appearance of haemagglutinin was consistently earlier with the irradiated material than with non-irradiated virus of equivalent residual infectivity. They could see no other interpretation than that of multiplicity reactivation in the sense that if 2 virus particles entered the same cell, each by itself having a 1 : 100 chance of initiating infection, the chance of infection being initiated would be considerably more than 2 : 100. Alternately, we might state that many cells which receive two "dead" virus particles can give rise to descendant virus particles. Though only limited experiments were done with heated virus, its behaviour was apparently identical with that of irradiated virus.

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In view of the Berry-Dedrick phenomenon (1936), in which active fibroma virus can be converted into myxoma by the addition of heat-inactivated virus and of Henle and Liu's work, it was of considerable interest to determine to what extent recombination might occur when one of the "parent" viruses had been inactivated by heat. The results to be reported indicate that recombinants can be derived from the interaction between an active and a heat-inactivated virus particle. Using the pairs MEL/WSE and M+/WS—, all four combinations of heated and active virus have given recombinants of the serological type of the heated component.

Materials and Methods.

The virus strains MEL, WSE, M+ and WS— used in all our recent work were again used exclusively (Burnet and Lind, 1952; Lind and Burnet, 1953). Heated virus was prepared by heating untreated allantoic fluid virus, from eggs harvested 1–2 days previously, at 56° C. for 30 minutes. WSE and WS— were completely inactivated as judged by failure of any infections to occur when the fluid was inoculated undiluted and diluted 10^{-1} and 10^{-2} into the allantoic cavity. The infectivity titre of MEL and M+ was greatly reduced from around 10^8 to between 10^0 and 10^1.

The experiments were carried out in de-embryonated eggs prepared as described in a recent paper (Burnet and Lind, 1954b), the usual procedure being to add 4·5 ml. of heated virus in appropriate dilution, and half an hour later the active virus in a volume of 0·5 ml. at 10 times the desired final concentration. One further hour was allowed for the adsorption of virus. The fluid was then discarded and RDE added (200 u. per ml.) for one hour. The preparation was washed three times, and then 5 ml. of glucose-penicillin-streptomycin Ringer solution (GPS Ringer) added. The fluids were harvested 6 to 7 hours after addition of the active virus.

These fluids were titrated for haemagglutinin (HA) and for serological character *in vitro* when the HA titre was adequate. Most attention was paid to the *in ovo* titration of the fluid in antiserum against the active component of the original mixture. This allowed the isolation of any recombinant of the heated component's serological type. As an example, we may take an experiment with heated M+ and active WS—. The first cycle fluids are titrated in anti-WS serum, which will reduce the titre of WS— to a level which will allow MEL recombinants to be isolated if they are present in sufficient number. Positive limit dilution fluids from such titrations were tested, as a routine, only for serological character and for the development of the indicator state against meconium inhibitor and sheep salivary mucoid, using methods recently described in detail (Burnet and Lind, 1954 a). Where indicated, other tests notably for pathogenicity for the chick embryo and for the mouse lung were sometimes used. For these tests fluids from a second passage at limiting infective dilutions were used.

Experimental.

The present series of experiments was initiated by an experiment designed to determine at what temperature M+ virus must be heated to destroy its capacity to react with WS— and give rise to MEL recombinants. Allantoic fluid virus was used unheated and heated for 30 minutes at 54° C., 56° C., and 58° C., respectively. Haemagglutinin and infectivity titres were respectively 800, 400, 200, 160 and $10^{8.5}$, $10^{6.5}$, $10^{3.0}$, $<10^{0}$. In the experiment active

WS — virus was used at a concentration equivalent to an HA titre of 20 while the M + was used at 1:40 (HA 20) for active virus and 1:20 for all the heated samples (HA 20, 10, 8).

The first cycle fluids obtained at 6½ hours after inoculation had the characters shown in Table 1. The haemagglutination tests were carried out in saline and in anti-MEL and anti-WS sera pretreated with KIO_4 as described in a preceding paper (Burnet and Lind 1954a).

TABLE 1.

Product of interaction of active WS — with heated M + virus.

Treatment of M + virus	Haemagglutinin Sal	aM	aWS	Infective titre in anti-WS serum 1:25
Unheated	80	12	8	MEL $10^{8\cdot0}$ M + $10^{8\cdot0}$
54° C. 30 minutes	50	32	2	MEL $10^{1\cdot8}$ M + $10^{2\cdot8}$
56° C. „ „	50	32	2	MEL $10^{1\cdot8}$ M + $10^{1\cdot5}$
58° C. „ „	60	40	2	MEL $10^{0\cdot8}$ M + Nil

Haemagglutinin: Titre shown when first cycle fluid titrated, Sal., with saline as diluent, aM., with anti-MEL serum 1:200, aWS with anti-WS serum 1:200 as diluent.

Infective titres: The values represent the approximate ID_{50} level with 0·05 ml. inocula, for MEL and M + viruses as judged by the characterization of fluids obtained in the two highest infective dilutions.

It will be seen that MEL fluids are produced in each case to about the same extent as M +, and with M + heated to 58° C. and containing no demonstrable surviving virus, only MEL was obtained.

This experiment indicated that in all probability M + virus particles, which had no power to initiate infection, could in some instances provide components for viable MEL recombinants. A more definite answer could be hoped for if recombinants could be recovered with a dose of unheated virus so small that there would be little chance of any active particle entering one of the few cells that had received a viable particle of M + virus. It is recognized that a reduction in infective titre from $10^{8\cdot5}$ to $10^{1\cdot0}$ does not necessarily or probably mean that the heated virus contains 10 normal living virus particles and $10^{8\cdot5}$ dead ones nor, alternatively, that it contains $10^{8\cdot5}$ uniformly damaged particles, each of which has a $10^{-7\cdot5}$ probability of initiating infection. It is not, however, experimentally possible to distinguish between the two alternatives or any intermediate, and the first is the more convenient way of discussing the situation.

TABLE 2.

Interaction of small doses of active virus with heated M +.

Heated M + (56° C. 30′)	Active WS —	Infective titre in anti-WS serum
1:10 (HA 16)	10^{-4} (HA 0·06)	MEL 10^{2} M + Nil
„	10^{-5} (HA 0·006)	MEL 10^{1} M + 10^{1}

Infectivity titre of undiluted heated M + = 10^{-1}.

In Table 2 are the results of an experiment in which M +, heated at 56° C. for 30 minutes and giving a nominal infective titre of $10^{1\cdot0}$, was diluted 1/10 and mixed with active WS— virus diluted 10^{-4} and 10^{-5}. Neither of the first cycle fluids obtained had a measurable haemagglutinin titre. If we accept these results at their face value and adopt the value of $10^{5\cdot6}$ as the ratio between ID_{50} and HA titres as expressed in our usual convention, and assume that there are 10^8 infectible cells in the allantoic cavity (Fazekas and Cairns, 1952), it is possible to make some numerical deductions.

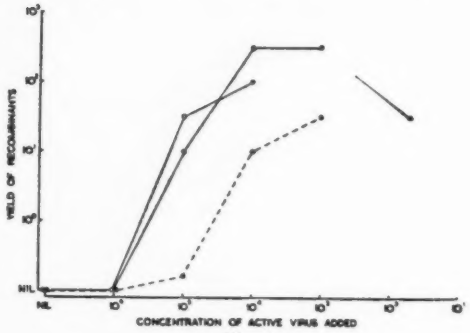

Fig. 1.

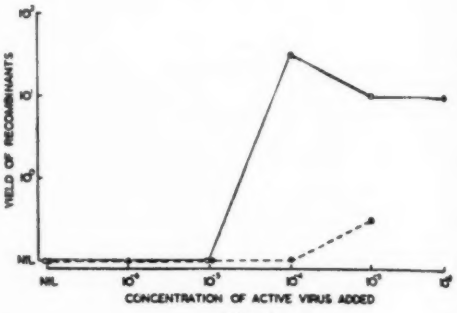

Fig. 2.

Fig. 1. Yield of recombinants in relation to the concentration of active virus added
O —— MEL from 3 experiments with heated M+/active WS —.
● ···· M + from heated MEL/active WSE.

Fig. 2. Yield of recombinants in relation to the concentration of active virus added
O —— WSE from heated WS —/active M +.
● ···· WS — from heated WSE/active MEL.

The first cycle fluid obtained from eggs inoculated with 5 ml. of a mixture containing a final concentration of heated M + 10^{-1} and active WS — 10^{-4} has a titre of 10^2 for MEL when titrated in the allantoic cavity (0·05 ml. standard inoculum), i.e., 10^4 MEL particles have been produced in the 5 ml. of fluid in the de-embryonated egg. The heated M + has a titre of 10^1 when titrated in the allantoic cavity. Therefore the inoculum for the de-embryonated egg would contain, in 5 ml. of 10^{-1} dilution of heated M +, 100 viable M + particles of which 50 might initiate infection. These 50 cells infected with viable M + would also be exposed to the possibility that an active WS — particle would enter. The WS — had an infectivity titre of $10^{8\cdot3}$. With an inoculum of 5 ml. containing this virus at a dilution of 10^{-4}, approximately 1/50 of the total available cells could receive a WS — virus particle. Thus we calculate that about 50 cells would receive viable M + while 1 only would receive both viable M + and WS —. Again keeping to our rather naive calculations and taking 100 virus particles as the approximate yield per infected cell, 5×10^3

M + particles might be expected from the cells singly infected with viable M +, and, allowing for recombination and partial interference, 20 MEL and 20 M + particles from the doubly infected cell. The actual result shows that there are more MEL type virus particles produced and many fewer M + than would be expected on the assumption that only "viable" particles are concerned. It is inescapable that some at least of the MEL fluids have been derived from M + particles which, in the absence of the active WS —, would never have initiated infection.

Similar experiments have been carried out with each component of the two systems M +/WS —, MEL/WSE used after heating to 56° C. for 30 minutes with graded doses of the second component in the active unheated state. The experiments all followed the general pattern of that reported in Table 2, the relatively large dose of heated virus (20 HA) being left in the membrane preparation for 30 minutes before the active virus was added. The results were sufficiently regular to allow the yield of recombinants of the same serological type as the heated virus to be plotted against the dose of unheated virus used. The impression given by Figs. 1 and 2 is that up to a certain concentration the yield is nearly proportional to the amount of unheated virus used, but beyond that point does increase further.

TABLE 3.

Production of recombinants from heat inactivated WSE or WS — virus.

Heat inactivated virus	Concentration of active virus	Infectivity titre and virus types from 1st cycle fluid when titrated with anti-MEL serum
WS —	M + 10^{-2} (HA 8)	$10^{1\cdot0}$ 2 **WSE**
	10^{-3}	$10^{1\cdot0}$ 2 WSE 1 M +
	10^{-4}	$10^{1\cdot5}$ 3 **WSE** 1 WS —*
	10^{-5}	Nil
	Nil	Nil
WSE	MEL 10^{-3} (HA 1)	$10^{1\cdot0}$ 2 WS— 2 MEL
	10^{-4}	$10^{1\cdot0}$ 4 MEL
	10^{-5}	$10^{0\cdot5}$ 4 MEL
	10^{-6}	10^{0} 1 MEL
	Nil	Nil

Heated virus used at concentration of 20 HA, haemagglutinin titre of lowest dilution of active virus shown in brackets. Recombinant strains are shown in heavy type.

* This strain (4351) showed a typical reaction and on transfer at limiting infective dilution, gave WSE types.

In these experiments both WSE and WS — were completely inactivated by 56° C. for 30 minutes in the usual sense that eggs inoculated with 0·05 ml. of virus undiluted and diluted 10^{-1}, 10^{-2} and 10^{-3} all showed no development of haemagglutinin after 3 days' incubation. The MEL and M + virus showed some persisting virus. In three experiments with M + the titre was reduced from around $10^{8\cdot5}$ to $10^{3\cdot0}$, $10^{1\cdot0}$, and in the third experiment one infection only

was produced in an egg inoculated with 10^{-2} dilution, probably as a manifestation of autointerference. In one experiment with MEL the infective titre was reduced from $10^{8.5}$ to $10^{1.7}$.

It is convenient to discuss first the experiments in which the virus was completely inactivated, and the results with heated WSE and WS — are shown in Table 3.

Three points emerge. The first is that the only type with WS serology to be obtained is the recombinant—the absence of viable original WS types is confirmed by their failure to appear in the first cycle fluids. One interesting partial exception is noted in the Table and is discussed at some length in a later section. Secondly, recombinants are not found below a certain dose of the active component and, finally, there is clear evidence of interference. In the absence of interference, the larger doses of active M + or MEL would have produced much more descendant virus than could be dealt with by 1 : 25 anti-MEL serum.

Table 4 gives the results of a similar experiment with heated MEL and active WSE. The production of M + recombinants runs parallel with the appearance of the analogous type in the other combinations.

TABLE 4.

Production of recombinants from heated MEL and active WSE.

Concentration of active WSE virus	Infectivity and virus types from 1st cycle fluid when titrated with anti-WS serum
10^{-3} (HA 0·7)	$10^{1.5}$ **4 M +**
10^{-4}	$10^{1.0}$ **6 M +**
10^{-5}	$10^{0.2}$ **1 M +** 3 MEL
10^{-6}	$10^{0.5}$ 3 MEL
Nil	$10^{0.5}$ 4 MEL

Heated virus (infectivity titre $10^{1.7}$) used at a final dilution of 1/45 (HA = 20).

Reciprocal recombinant in heated M +/active WS — experiment.

It is obviously more difficult to detect the recombinant whose serological character corresponds to that of the active component, and only in one of the heated M + active WS — experiments has this been done. The method used was to mix the first cycle fluid with anti-MEL serum to inactivate any M +, and test by inoculation onto the chorioallantois (CA) for the presence of WSE, which has the power to kill the embryo with haemorrhagic lesions in brain muscles or lung when inoculated by this route.

It will be seen from Table 5 that WSE is found only with the largest amount of active WS — and in much smaller amount than MEL.

TABLE 5.

Test for WSE in first cycle fluids from heated M +/active WS — experiments.

Concentration of active WS — virus used with M + to give the first cycle fluid to be tested	Effect on embryo on CA inoculation of first cycle fluid diluted in anti-MEL		Titre WSE	Titre of MEL by allantoic inoculation
	10^{0}	10^{-1}		
10^{-3}	6,2,0,0	5,5,2,0	10^{1}	$10^{2\cdot5}$
10^{-4}	1,1,0,0	1,0,0,0	Nil	$10^{2\cdot5}$
10^{-5}	0,0,0,0	0,0,0,0	Nil	$10^{1\cdot0}$
10^{-6}				Nil

Scores for lesions are based on 1 for death of embryo at four days, 2 for death at 3 days, and 1 to 3 for lesions in each of brain, pectoral muscles and lung, maximum score being 11. Dead embryos without other lesions are regarded as negative.

Other characters of recombinant strains.

Most of the recombinant strains obtained in these experiments were also tested by inoculation, after limit dilution passage, into mice intranasally and on to the chorioallantois. No anomalies were observed with any of the MEL or M + strains, but some interesting features were observed with the WS recombinants which were all from nominally dead virus of that serological type. All showed full pathogenicity for the mouse lung, the lowest score recorded being 3·8, and the deviations were all in regard to tests made on the chorioallantois.

A typical result after inoculation of WSE virus diluted to a titre of 1 AD is death of the embryo at about 60–70 hours, with gross haemorrhage in brain and muscles, and smaller close set haemorrhagic lesions in the lungs. With WS —, eggs opened 4 days after inoculation show embryos either alive or recently dead without haemorrhages or other macroscopic lesions. M + lesions correspond to those of WSE, MEL to those of WS —. In order to give a quantitative basis we have scored the lesions one to three plus in brain, pectoral muscles and lungs with a maximum of 9 and added two if death occurred before 72 hours, one if between 72 and 96 hours. On this scale stock WSE gave lesions averaging 6·7, and stock WS — averaged 0·4. The score is influenced to some extent by variations in susceptibility amongst the embryos. Very rarely an embryo survives 4 days with WSE, and even more rarely an embryo dead after WS — will show slight muscle haemorrhages.

Using the recombinants obtained from heated virus of the same serological type, the figures shown in Table 6 were obtained.

The Table brings out the point that, particularly for WSE, the range of pathogenicity level is much wider for recombinant strains than for stock virus. The same holds though to a much more limited extent for WS — strains. It should be pointed out that a similar range of pathogenicity for the chick embryo

is found in M + recombinants from MEL/WSE experiments, but, for obvious reasons, the two strains M + and WS — chosen for passage and use as stock viruses had sharply defined and reproducible characters in this regard.

TABLE 6.

Range of pathogenic action on chick embryo of WS type viruses of different origins.

Source of viruses	No. of embryos showing lesion scores										Mean
	0	1	2	3	4	5	6	7	8	9	
WS — from heated WSE	22	21	6	4	—	—	—	—	—	—	0·83
WSE from heated WS —	—	1	—	3	3	4	3	4	1	—	4·8
Stock WSE	1	—	—	—	—	1	1	3	3	2	6·7
„ WS —	18	3	2	1	—	—	—	—	—	—	0·42

The only major anomaly in the series of experiments was from an experiment with heated WS — plus active M +. This gave by *in vitro* characterization 6 WSE strains and 1 WS — strain.

All the WSE strains had typical reactions on mouse lung and chorioallantois, i.e., their structure could be expressed according to the formula used in previous papers as adf — ce. The strain (4351) with the WS — reactions ad — C was titrated, and at the limit of infectivity gave 4 WSE (ad — c) strains and 1 (4798) with *in vitro* WS — reactions but typical pathogenicity for the chick embryo its formula therefore being adf — Ce. This was again taken to limit dilution and gave 3 WSE strains and one (6139) with WS — characters. Fluid 6139 was passed again at limit dilution, and this time all three fluids at limiting dilution were of WS — character *in vitro*.

When tested on the chorioallantois, two gave the results to be expected from WS — strains with scores 2,2,1,0 and 1,1,0,0. The third strain gave series 4,3,3,2, three embryos having a few definite haemorrhages in the pectoral muscles. The intensity of the lesions was much less than with stock WSE strains or with the recombinant WSE strains.

In this series we have a very interesting situation which will need to be covered in any interpretation of influenza virus genetics. The initial recombinant 4351 came from an experiment in which the heated WS — component gave no evidence of viability. All the other strains obtained with WS serology were of the combinant WSE type to be expected. 4351 is not a typical WS — since on two consecutive limit dilution passages strains that are *in vitro* WSEs predominate, but by following the WS — descendants we have what appears to be a gradual return to the full WS — character.

In view of the recent demonstration by Perry and Burnet (1953), van den Ende and Perry (1954), that between two influenza B strains there is a striking transferability of the capacity to produce mouse lung lesions, it is probably

desirable to place on record the absence of any such transfer amongst the A strains that we have used. Table 7 shows the values obtained with recombinants from the heated virus experiments.

TABLE 7.

Pathogenicity of recombinant types for mouse lung.

Recombinant type	Average mouse lung lesions
MEL	1·7, 1·6, 1·5, 1·5, 1·25
M +	2·2, 2·0, 1·4, 1·4, 1·0, 0·8, 0·7, 0·5
WSE	5, 5, 4·7, 4·5, 4·4, 4, 3·8
WS —	5, 5, 5, 4·5

Each figure is the mean score of 4 mice each inoculated with 1AD of an individual fluid. 5 = death on the 5th day or earlier. 4 = death on 6th or 7th day. 0–3·5 = degrees of lung consolidation when killed at 7 days.

No specific tests were made in regard to our factor Bb (heat stability of the haemagglutinin) owing to its probable complexity (see Burnet and Lind, 1952). The Dd character was always tested and gave consistent findings, all heated MEL and M + fluids showing no inhibition of haemagglutination by sheep salivary mucoid; WSE and WS — strains were both inhibited, the former to higher mucoid dilutions than the latter. The results can be summarized that, except for some variation in the expression of character Ee, all the recombinants conformed to standard patterns:

MEL, ADF — CE: M +, ADF — ce: WS —, adf — CE: WSE, adf — ce.

DISCUSSION.

In the main series of experiments a dose of heat inactivated virus was given, which on the basis of its haemagglutinin titre, must have allowed more than one particle to be taken up by all accessible cells. Half an hour later small doses of active virus, potentially capable of recombination, were added and given the opportunity to enter already occupied cells. A dose of the order of 5 HA units, about 1:100 dilution of an average allantoic fluid virus, is approximately what is required to provide one virus particle for every cell. Virus diluted to higher levels will only be able to infect a corresponding fraction of the available cells. It is reasonable to assume a random distribution of active particles uninfluenced by what has previously entered the cell. If all cells are already occupied by one or more inactive particles, the fate of a given active virus particle after entering a cell may be one of three. (1) Interference may prevent any demonstrable liberation of virus. (2) Recombination may occur by which a component of the active virus replaces a component of

the inactive virus in the same cell. (3) Normal multiplication of the active virus takes place with liberation of progeny.

The experimental findings that have been reported show that as would be expected there is a high degree of interference, a rough estimate indicating that somewhat less than 1/1,000 of the cells receiving active virus contribute anything to the pool of virus harvested at 6–7 hours. The smallest degree of interference was seen in the heated WSE/active MEL experiment, all the mixtures showing MEL strains despite the use of anti-MEL serum.

If we confine our attention for the time being to experiments with completely inactivated WS — and WSE, we are forced to adopt the hypothesis that some genetic material from the inactivated virus can combine with material from the active virus to give rise to viable units. As in all our previous work, we have found that recombination in the present experiments involves only certain blocks of characters. We may express the structure of MEL and WSE, using the symbols previously adopted (Burnet, Fraser and Lind, 1953) as ABDF — CE and abdf — ce. The results of the recombination experiments with heat inactivated virus agree with the hypothesis that we are dealing with two sets of linkage groups which multiply independently in the cell. By the use of borderline heat treatment, it is possible to render one linkage group non-viable without equivalent damage to the other in a proportion of virus particles. Taking Aa and Cc as symbols for the two sets of linkage groups, we can tabulate the possibilities as follows in terms of the percentage of virus types to be expected from a doubly invaded cell.

TABLE 8.

Percentage of different virus types expected to be produced in cells doubly invaded by an active MEL particle and active or heat-damaged WSE.

	MEL AC		×	WSE ac	
	AC MEL	Ac M +		aC WS —	ac WSE
Active WSE					
ac	25	25		25	25
Damaged WSE					
ac	50	0		50	0
ac	50	50		0	0
ac	100	0		0	0

This provides an explanation of the most important feature of our results, the recovery of the serological type of the heat-inactivated virus only in the form of the recombinant and, subject to one anomalous result already discussed, never in the original type. The failure to find any dissociation of characters within either linkage group in this series of experiments provides rather strong

evidence in favour of the hypothesis that the linkage group, not the virus nor the individual gene, is the normal unit which replicates intracellularly.

Within the limits of this study the behaviour of heat treated virus is intelligible on the basis of the hypothesis that two genetic units, which we have called linkage groups, are alone involved. We have already unpublished evidence that the simple postulation of two linkage groups is inadequate to explain the results of recombination experiments, particularly where characters involving virulence are concerned. Nevertheless, in the MEL–WSE system we have virtually no alternative to this hypothesis, and only a minor qualification needed to incorporate results of the type shown in Table 8 is required. It is tempting to consider that the linkage groups are also the units involved in interference phenomena, but discussion of that phase must await fuller experimentation.

If, as seems likely (Burnet and Lind, 1954b), most influenza virus particles (spheres and filaments) have more than one set of genetic determinants, the position becomes proportionately more complex, but no modification of principle is involved.

SUMMARY.

In the recombination systems MEL/WSE or M +/WS — heat inactivation of one component (56° C. for 30 minutes) does not prevent the appearance of recombinants of the same serological type as the heated component.

With only very minor exceptions, all the results obtained are compatible with the hypothesis that the genetic material of the viruses is distributed in the form of two linkage groups.

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RECOMBINATION OF INFLUENZA VIRUSES IN THE DE-EMBRYONATED EGG

1. THE USE OF PERIODATE-TREATED SERA FOR *IN VITRO* CHARACTERIZATION OF INFLUENZA VIRUS STRAINS

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With the demonstration that recombination of influenza viruses could occur in the allantoic cavity (Burnet, Fraser and Lind, 1953), it became desirable to seek an even simpler technical method for such experiments. The main labour involved was in the repeated washings of the allantoic cavity that were called for. The use of the de-embryonated egg, introduced by Bernkopf (1949), seemed an obvious answer to this problem provided the method were otherwise suitable. This has in fact proved to be the case, and for some time all our recombination experiments have been made in this preparation.

The possibility of demonstrating the presence of recombinants in first cycle fluids by *in vitro* studies has also been explored. In the experiments recently reported allantoic titration of first cycle fluids indicated that, in the mixtures we employed, infective virus particles of MEL, M +, WSE, and WS — types were present in numbers of at least the same order of magnitude (Burnet, Fraser and Lind, 1953). If the total amount of haemagglutinating virus (complete and incomplete) in the first cycle fluid shows the same ratio between the types as in infectivity titrations, it should be possible to determine the proportion of each by *in vitro* tests, using unheated fluid for anti-haemagglutinin tests with anti-MEL and anti-WS sera and heated fluid against appropriate mucoid inhibitors with and without antisera. To make such *in vitro* tests practical, it is necessary (a) that the sera used shall be highly specific, and (b) that they contain no non-specific mucoid inhibitor of haemagglutination by heated virus.

After a number of preliminary tests with RDE-treated sera and sera appropriately absorbed with virus on PVR cells (Fazekas, 1949), attention was turned to the use of periodate recently recommended by Davoli (1952). This has proved highly satisfactory and the present paper is concerned with the use of periodate-treated immune rabbit sera with or without known amounts of semi-purified meconium inhibitor for the characterization of mixed fluids containing MEL, WSE, M + and WS — viruses.

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EXPERIMENTAL.

The influence of graded amounts of potassium periodate on immune rabbit sera.

A standard volume 0·25 ml. of each serum R803, anti-M +, R806 anti-WS —, was mixed with the volumes shown of M/100 KIO_4. After mixing at room temperature, the tubes were placed overnight in a refrigerator and the following morning each was made up to 2·5 ml. (i.e., 1 : 10 serum dilution) by the addition of equal parts of saline and glycerol. The glycerol had the dual function of inactivating any residual periodate and serving as a preservative. To the first tube, which had already the full volume, one drop of 10 p.c. glycerol was added to inactivate any periodate.

Following Fraser (1953), we found that the most convenient way to use immune sera for the characterization of virus mixtures was to maintain a constant concentration of antiserum by using a 1 : 200 dilution of serum (treated or untreated) in saline as the diluent in which serial dilutions of the virus or virus mixture were made. When, as is usual, a series of 2-fold dilutions was employed, the first tube of the series contained as diluent 1 : 100 serum, all subsequent ones 1 : 200. When the virus was added to the first tube and serial transfer carried out, all tubes finally had a concentration of 1 : 200 antiserum.

Diluents containing 1 : 200 or 1 : 100 of serum treated with each level of periodate were prepared and several viruses titrated in each. These included heated viruses MEL and WSE grown in de-embryonated eggs, active allantoic fluid viruses M + and WSE, as well as heated LEE to detect any residual inhibitor. The titres obtained are shown in Table 1.

It will be seen that gross excess of periodate damages the homologous neutralizing activity, while below a certain level non-specific inhibitor is active.

TABLE 1.

Haemagglutinin titres of viruses titrated in dilute immune sera treated with graded amounts of periodate.

Volume ratio of M/100 KIO_4 to serum	Serum anti-MEL 1:200				Serum anti-WS 1:200			
	H MEL	H WSE	M +	H LEE	H MEL	H WSE	WSE	H LEE
9:1	1	14	10		48	8		
7:1	< 2	18	7		60	5		
5:1	< 2	22	6		48	6		
3:1	< 2	20	3	5	44	2	30	5
2:1	< 2	22	3	5	48	< 2	22	5
1:1	< 2	6	3	< 2	48	< 2	15	< 2
No. KIO_4	< 2	3	3	< 2	40	< 2	15	< 2
Saline alone	44	22	480	5	44	22	800	5

All serum periodate mixtures were made up to a final serum dilution of 1:10 by the addition of saline and glycerol.

H = heated virus, MEL and WSE 52° 30 minutes, LEE 56° 30 minutes.

Optimal action is obtained where 2 volumes of M/100 periodate are added to one volume of immune rabbit serum. This ratio has been found generally applicable to immune rabbit and ferret sera, and the routine procedure has been to mix 5 ml. of serum with 10 ml. of M/100 KIO_4, allow to act overnight at 4° C., and then add 10 ml. sterile glycerol. This gives a 1:5 stock dilution in a convenient form. Diluting fluids for titrations are prepared by making a 1:40 dilution of stock serum in saline (1:200 serum) with a smaller amount of 1:20 (1:100 serum) to be used in the first tube of a series of two-fold dilutions.

The in vitro *differentiation of four virus types.*

In order to allow a clear differentiation of the four types of virus involved in our standard recombination experiments, it is necessary to make use of diluents containing a known amount of a mucoid inhibitor as well as antiserum and to use heated virus. We have used as inhibitor semi-purified meconium as prepared by Curtain, French and Pye (1953). The most convenient dilution is 1:200 of our stock 0·2 p.c. solution of the semi-purified material in 50 p.c. glycerol saline. This is highly inhibitory for heated WSE and M +, but has no action on heated MEL or WS —.

Table 2 illustrates the reactions of typical allantoic fluids from pure clones of the four strains heated for 30 minutes at 52° C.

TABLE 2.

Reaction of heated viruses with treated sera and inhibitor.

Diluent	HA titres of heated viruses			
	MEL	M +	WS —	WSE
Saline	480	400	700	480
Anti-MEL (R803/200)	3	< 2	500	280
Anti-WS (R806/200)	450	400	10	4
Anti-MEL + meconium	3	< 2	320	2
Anti-WS + meconium	160	< 2	320	< 2
Meconium 1:200	320	< 2	280	< 2

The results show slight variations from one batch of fowl cells to another in the degree to which a virus titre is reduced by the heterologous serum. With different cells the values may range from 50 to 120 p.c. of the titre given in saline, but are never less than 50 p.c. (1 tube difference) of the saline value. In general, a MEL or M + anti-serum has a slight effect on a WS strain, but a WS type anti-serum none on a MEL-type strain. It may be useful to tabulate the percentage to which the titre of each virus is reduced by the different reagents.

Ideally the method should determine how much of MEL, M +, WS — and WSE virus types are present in any mixture, but in fact the information will always be more limited.

TABLE 3.

Residual titres shown on titration in special diluents.

Reagent	MEL p.c.	M + p.c.	WSE p.c.	WS — p.c.
Homologous serum	0·6	< 0·5	1·0	1·4
Heterologous serum	70–100	70–100	50–80	50–80
Meconium	60	< 0·5	< 0·5	40–60

The existence of anomalous neutralization described below makes it impossible to analyse the content of the first cycle fluid as if it were a simple mixture, and we have not made much practical use of the titrations of heated virus with anti-sera and inhibitor. On a number of occasions when M +/WS — mixtures were used, it was possible to show that the residual titre obtained when a first cycle fluid, with a haemagglutinin of 100–200, was titrated in anti-WS serum plus inhibitor was in the range 4–10. On addition of 1:200 anti-MEL serum to the tubes showing this agglutination, it was eliminated. This behaviour could be taken as direct evidence of a proportion of MEL recombinants in the fluid. In practice we have found it preferable to isolate MEL strains by inoculation into chick embryos in the presence of anti-WS serum. The remainder of this paper is therefore concerned with the difference between the serological behaviour of artificial mixtures of two viruses and of first cycle fluids.

Behaviour of artificial mixtures.

Mixtures of two viruses of different serological type prepared from allantoic fluids or from the fluids from de-embryonated egg cultures give the results to be expected from the individual behaviour of their components. Some examples are given in Table 4 from an experiment in which fluids from de-embryonated eggs were used. In view of the possibility that heating a mixture of two viruses to 52° C. might cause some aggregation or other abnormal behaviour, two sets of mixtures were used. One set was of viruses heated separately, the other mixtures of active fluids were then heated under the standard conditions and titrated as shown in the Table.

TABLE 4.

Behaviour of artificial mixtures of heated viruses and of mixtures of active viruses subsequently heated.

Heated viruses	Sal	aM	aWS	Mixtures	Pre-heated Sal	Pre-heated aM	Pre-heated aWS	Heated after mixing Sal	Heated after mixing aM	Heated after mixing aWS
MEL	40	<2	40	MEL/WSE	64	8	40	60	8	24
WSE	32	12	<2	MEL/WS—	60	12	40	64	12	32
M+	30	<3	30	M+/WSE	24	6	16	32	6	8
WS—	48	28	2	M+/WS—	40	8	32	60	16	20

Figures represent haemagglutinin titres of titrations made in the three standard diluents, saline (sal), periodate-treated anti-MEL rabbit serum (aM) 1/200 and anti-WS serum (aWS) 1/200. All mixtures were of equal volumes of the fluids whose titres after heating are shown in the first section of the table.

As a working rule we regard any titration as showing normal behaviour if the sum of the titres given in anti-MEL and in anti-WS sera is 50 p.c. or more of the value given in saline. It will be seen that in the Table only one mixture falls slightly below this level.

Double neutralization in first cycle fluids showing recombination.

The most interesting use of these reagents is in connection with the phenomenon of "double neutralization," which has already been briefly referred to in communications from this laboratory (**Fraser**, 1953). Any first cycle fluid from WSE/MEL or M +/WS — mixtures which shows a good yield of recombinants has, in our experience, given haemagglutinin which is significantly neutralized by *both* anti-MEL and anti-WS sera. The genetic significance of this phenomenon, as well as its practical use in recombination experiments, will be dealt with in the next paper of this series. Here we are only concerned with the behaviour *in vitro* of typical fluids showing the phenomenon.

We may take, for instance, two fluids from experiments referred to in the next paper, 96508-9 and 96972-3, from recombination experiments in de-embryonated eggs in which M + and WS — were used as parent viruses.

TABLE 5.

Fluids from recombination experiments showing typical "double neutralization."

First cycle fluid	Saline	HA titres in aM	aWS	Combined percentage
96508–9	80	12	8	25
96972–3	200	10	4	7

The combined percentage is the sum of the titres in aM and aWS in terms of the original HA titre.

It can readily be shown that the capacity to neutralize such haemagglutinin is confined to the two specific sera concerned. Ferret sera covering the main subtypes of influenza A were kindly provided by Mr. J. White and treated with periodate. Titrations of WS —, M + and fluid 96508-9, all from de-embryonated eggs, were made in saline and suitably diluted immune sera.

TABLE 6.

To show the specificity of the double neutralization.

Diluent	M +	WS —	96508–9
Saline	100	80	80
Anti-MEL	*2*	80	*16*
Anti-WSE	80	*2·5*	*6*
Anti-SW	80	80	80
Anti-CAM	80	80	80
Anti-CHO	64	70	64

Figures show HA titres when viruses were titrated in the diluents shown. CAM and CHO are A strains typical of Australian influenza in 1946 and 1951 respectively.

The anomalous haemagglutinin is somewhat more readily inactivated by heat than similar preparations of pure M + or WS —haemagglutinins. No change in the double neutralization occurs up to the point of destruction.

TABLE 7.

Effect of heat on haemagglutinin showing double neutralization.

		Saline	anti-MEL	anti-WS
M +	Unheated	140	< 2	80
	56° 30 min.	20	< 2	20
WS —	Unheated	160	140	3
	56° 30 min.	10	8	< 2
M +/WS —	Unheated	200	10	5
96972–3	54° 30 min.	64	4	3
	56° 30 min.	4	< 2	< 2

The effect of treating anomalous haemagglutinin at various pH levels was tested by mixing equal volumes of standard buffers of 0·1 ionic strength and virus fluid 96972-3. These were held at 37° C. for 1 hour and then titrated in the three diluents shown.

TABLE 8.

Effect of pH on anomalous neutralization with fluid 96972–3.

Buffer	pH	Sal	anti-M	anti-WS	Percentage
Acetate	5	< 2	< 2	< 2	
Phosphate	6	50	16	5	42
"	7	120	10	5	12·5
"	8	160	10	4	9
Veronal	8	140	4	3	5
"	9	160	5	3	5
Glycine	10	120	4	< 2	3·3
"	11	80	< 2	< 2	< 2

It will be seen that double neutralization becomes progressively more marked with increasing alkalinity. This is also shown with homologous neutralization, particularly with WS— and anti-WS, and cannot therefore be regarded as necessarily indicating any dissociation of the doubly neutralizable haemagglutinin.

DISCUSSION.

This communication is a preliminary to papers specifically concerned with recombination of influenza viruses in the de-embryonated egg and only the technique of *in vitro* testing of mixtures and its implications will be discussed. For the limited purpose of analysing fluids containing one or more of the types MEL, M +, WSE, WS —, the periodate-treated sera we have described serve

admirably. A somewhat more superficial survey over a wider range of strains indicates that periodate treatment does not render sera suitably specific for the more recently isolated A prime strains CAM 1946 (the first A prime to be recorded in the literature) and CHO 1950 (which is serologically identical with the L subtype of European influenza 1950-51). Haemagglutinin of these strains is inhibited to relatively high titre by periodate-treated normal or unrelated immune sera.

It will be seen from the data presented that two *in vitro* qualities may be used as an indication of the occurrence of recombination. The most interesting and, as will be shown subsequently, most reliable is the occurrence of double neutralization. This has already been used for this type of work by Fraser (1953). With reagents such as we have used, we consider that any type of simple mixture of MEL and WS virus will give titres with 1 : 200 anti-MEL and 1 : 200 anti-WS which, when added together, give a value more than 50 p.c. of the titre in saline. Any value below 25 p.c. is regarded as unequivocal evidence of recombination.

The second is the recognition of meconium and serum resistant agglutination not to be accounted for on the composition of the original mixture. When this has been observed we have been able to isolate the corresponding recombinant from the fluid. In general, however, it is more satisfactory to use isolation techniques.

For experiments aimed at determining the conditions under which recombination occurs, estimation of the extent of double neutralization and detection of the dominant recombinant by isolation in the presence of heterologous antiserum provides a relatively rapid and convenient technique.

SUMMARY.

A method of treating influenza immune sera with periodate to destroy inhibitors other than specific antibody is described.

The use of such sera for the analysis of mixtures and first cycle fluids from recombination experiments is described.

First cycle fluids from recombination experiments using the serologically distinct M + and WS — parent strains contain a high proportion of virus haemagglutinin neutralized by both antisera.

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RECOMBINATION OF INFLUENZA VIRUSES IN THE DE-EMBRYONATED EGG

2. THE CONDITIONS FOR RECOMBINATION AND THE EVIDENCE FOR THE POSSIBLE EXISTENCE OF DIPLOID INFLUENZA VIRUS

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In previous papers (Burnet, Fraser and Lind, 1953; Lind and Burnet, 1953) the production of recombinant influenza virus strains in the allantoic cavity has been described. The technical difficulty of adequately washing out the cavity at the required stages has led to the substitution of the de-embryonated egg preparation. In this paper we describe the methods used and indicate its application to a study of the conditions under which recombinants are produced when mixtures of M + and WS — are used.

MATERIALS AND METHODS.

The virus strains used were the stock strains MEL and WSE and the two recombinants previously described M+ and WS— (Burnet and Lind, 1952). These strains have been repeatedly reisolated from limiting infective dilutions and are completely stable in their behaviour. Stock material was prepared by inoculating 11-day embryos two days before the beginning of the experiment.

De-embryonation of chick embryos. The embryos were normally used on the 15th day of incubation, but occasionally 14 or 16-day embryos were used. The technique was modified from that of Bernkopf (1949) to allow the retention of most of the visceral layer of the allantoic membrane. If this is present, the titres obtained are approximately twice as high as in preparations with only the chorioallantois. The egg is drilled around the base of the air space and the cap removed. The shell membrane and chorioallantois forming the floor of the air space are removed and most of the allantoic fluid poured off. A slit is now made in the amnion with scissors, and this is extended for an inch or more so as to cut the thin allantoic membrane lying over the yolk sac. Care is taken not to damage the yolk sac. The embryo is now drawn out of the amnion and the umbilical stalk cut. The yolk sac is very gently pulled out through the large slit in the allantois, which slides back to form a crumpled cuff when the operation is completed. When a good deal of the yolk sac has been freed, the egg is tilted over a receptacle for waste and the yolk sac allowed to "flow" over the cut edge of the shell. By rolling the egg from side to side and easing back the allantois

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at any point where it holds back the yolk sac, the latter can be wholly freed and needs only a small cut of the scissors where it is attached to the albumen sac to release it. A slit is now made in the allantois overlying the albumen sac so that the contents can fall out easily. The shell now contains, in addition to the chorioallantois lining, the shell membrane, a tassel of membranes including the allantois and the amnion, all the superficial cells of which are exposed to contact with the fluid inoculum.

The preparations are washed twice with about 10 ml. of warm Ringer solution, and are capped with sterile aluminium milk bottle caps attached to the egg shell by a strip of adhesive tape over the centre, which combines the function of a label, a hinge and a fastening.

Diluent. The basic diluent for all inocula is standard Ringer solution (NaCl 0·85 gm., KCl 0·025 gm., $CaCl_2$ 0·03 gm., made up to 100 ml. with distilled water) to which glucose, penicillin and streptomycin are added to give a final concentration of 0·1 p.c. glucose and approximately 20 units of penicillin and 70μg. of streptomycin per ml. This is referred to as GPS Ringer.

Inocula. Infected allantoic fluid virus is diluted in GPS Ringer, usually to a haemagglutinin titre (HA) of 20, and 5 ml. added to each de-embryonated egg. When a mixture of two viruses is used as inoculum, 2·5 ml. of each virus dilution are placed in the egg.

The de-embryonated eggs are supported in holders on a rotating disc at an angle of 15° to the horizontal, the long axes of the eggs being parallel to the shaft. The whole is contained in an incubator maintained at 37 ° C. and with a humidity of 60–70 p.c. The disc rotates once a minute, allowing the 5 ml. of fluid to bathe all the membranes. The amount was chosen as the smallest convenient volume which would be adequate to keep the whole system constantly bathed under these conditions.

In all standard experiments the inoculum is removed at 1 to 1½ hours and replaced by 5 ml. of GPS Ringer containing 200 units of RDE per ml. The eggs are returned to the incubator for an hour to allow removal of all superficial receptors and liberation of any virus which has been adsorbed but failed to induce infection. The fluid is then removed and the cavities washed out three times with standard Ringer solution before being refilled with 5 ml. of GPS Ringer. Haemagglutinin appears between 3 and 4 hours after the first addition of virus, and the content of the eggs is usually harvested after 6–8 hours.

In many experiments the harvested fluid was replaced by another 5 ml. of GPS Ringer and the incubation continued overnight. When harvested at 22–23 hours, the fluid has a high virus titre and is moderately turbid with desquamated cells.

Analysis of first cycle fluids. All fluids harvested, whether at 6–8 hours or overnight, are titrated for their content of haemagglutinin and its serological character by the method described in the preceding paper. This involves titration of the fluid in three diluents, saline, anti-MEL rabbit serum diluted 1:200 in saline and anti-WS serum similarly diluted, both sera having been treated with an optimal amount of periodate to destroy nonspecific inhibitors of haemagglutination.

Isolation of recombinant strains was by the culture of limiting infective dilutions of the first cycle fluid in 11-day embryos inoculated into the allantoic cavity. Usually a preliminary titration was made, and on the basis of this a large number of embryos inoculated with two dilutions close to the limit of infectivity, e.g., $10^{6\cdot5}$, $10^{6\cdot0}$ of first cycle fluid stored in the interim in a dry ice cabinet. Each virus positive fluid was tested for serological type and for conversion to indicator on heating to 52° C. for 30 minutes. The procedure was as follows: Portion of the allantoic fluid was heated to 52° C. for 30 minutes and both heated and unheated virus titrated for haemagglutinin. Five agglutinating doses (ADs) of unheated virus were tested against a 1:200 dilution of each anti-serum, and 5 ADs of the heated virus

against 1:2,000 of the two stock inhibitors, semi-purified specimens of meconium and sheep salivary gland inhibitors. All these reagents were used at the dilution showing the sharpest differentiation between the types.

TABLE 1.

Virus type	Unheated			Heated		
	Saline	anti-M	anti-WS	Saline	Mec.	Sheep M.
MEL	+	—	+	+	+	+
M +	+	—	+	+	—	+
WSE	+	+	—	+	—	—
WS —	+	+	—	+	+	±

+ haemagglutination. — no haemagglutination.

Table 1 shows the primary differentiation of the four types of virus being considered. In many experiments all that was attempted was to show whether any recombinant MEL strains could be isolated from the first cycle fluid when WS strains were largely eliminated by using anti-WS sera. The technique was to titrate dilutions made in anti-WS rabbit serum diluted 1: 25 in normal horse serum saline. In most experiments six eggs were inoculated with a single 1: 100 dilution of the first cycle fluid. The situation can be visualized from Table 2, which shows the titres of M + and WS — in control and antibody containing diluents.

TABLE 2.

Effect of dilution in anti-serum on infectivity titres of M + and WS — viruses.

Virus	Diluent		
	Serum saline*	Anti-MEL/25	Anti-WS/25
M +	$10^{-9\cdot5}$	$10^{-4\cdot7}$	$10^{-9\cdot5}$
WS —	$10^{-8\cdot8}$	$10^{-9\cdot7}$	$10^{-5\cdot5}$

Values are ID_{50} titres from titrations in the allantoic cavity.

* Saline containing 10 p.c. horse serum.

In a mixture with roughly equal amounts of WS and MEL type viruses, titration in anti-WS will reduce the WS titre by 3 logs and leave only MEL types in the significant dilutions. When MEL and M + are present in approximately equal numbers, MEL will predominate in the final fluid. Thus if MEL strains are present they will be detected unless they are very heavily outnumbered by other types. In several experiments fluids from M + infections alone were similarly tested without finding any variants with MEL reactions.

EXPERIMENTAL.

The course of liberation of haemagglutinin in the standard preparation.

There is an earlier liberation of virus in our preparations than is described for the allantoic cavity. After the three washings at 2 hours the fluid contains no trace of haemagglutinin even when one-fourth of a unit is tested for by adding 0·25 ml. of red cells to 1 ml. of the undiluted fluid. This makes it easy to

detect the first liberation of haemagglutinin which occurs at 3 hours. Moderate amounts (HA titre 10–40) are always present at 4 hours. The time of appearance is almost the same for MEL, M +, WS — and mixtures of these, but WSE is somewhat delayed. Fig. 1 is designed to illustrate the sequence of washings, etc., and to show the changing levels of HA in the fluid for an experiment in which M + was used.

The influence of concentration of virus in the inoculum on recombination.

Theoretically recombination should only be expected when a considerable proportion of cells receive one or more particles of each virus. This will only occur with any frequency when the total number of virus particles is equal to or greater than the number of available cells. If the preparation has about 10^8 cells as suggested by Fazekas and Cairns (1952), then 5 ml. of virus with a HA titre of 2·5 should just suffice to provide on the average one virus particle for each cell. This allows $10^{6\cdot3}$ ID_{50} per HA unit and accepts the ID_{50} as corresponding approximately to one particle.

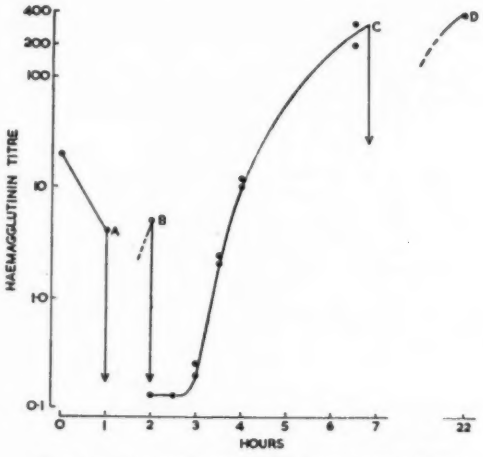

Fig. 1. The sequence of haemagglutinin levels in a standard experiment with the de-embryonated egg. The initial inoculum has a HA titre of 20: after 1 hour when the fluid is removed the level has fallen to A. At this point RDE is added and virus corresponding to B is liberated from cell surfaces, etc., in 1 hour. At this stage the cavity is thoroughly washed and no HA is detectable. The subsequent rise in HA to 6½ hours is shown, two series of experiments being combined. At C the eggs were drained and refilled, a final overnight harvest being taken at D.

Several experiments on the dosage of inoculum required to provide recombinants have given similar results; the following experiment is the most complete of the group. An equivalent M +/WS — mixture was diluted in 0·5 $\log_{10}$ steps and 5 ml. amounts in GPS Ringer tested each in 2 eggs. The fluids from each pair of eggs were pooled and tested in the fashion described under methods, the first fluids being harvested at 8 hours. The results are tabulated in Table 3, A and B.

These Tables present a number of points of interest. In the first place it will be seen that "double neutralization" is well shown in the first three dilutions, but not in the last two with initial HA titres of 0·8 and 0·25. The yields of virus in the first 8 hours and that of the next 15 or 16 hours closely

resemble each other in quality, and it will also be noticed that the titres of the pools from the two smaller inocula are significantly lower in both samples than those of the first three sets. These two points favour our contention that the use of RDE renders cells not initially infected unavailable for subsequent infection. Both yields can be regarded as essentially first cycle fluids from the same cells. We would conclude that, under the circumstances of this experiment, 5 ml. of virus with HA titre of 2·5 is sufficient to infect nearly every cell and to produce a large proportion of double infections with both M + and WS —.

TABLE 3.

Influence of dilution on production of recombinants by M +, WS — mixtures.

A. In vitro *characters of first cycle fluids.*

H.A. of inoculum		8-hour fluid			22-hour fluid			Percentage*	
		Sal.	Anti-M	Anti-WS	Sal.	Anti-M	Anti-WS	8 hr.	22 hr.
M +/WS —	25	160	4	3	200	10	4	4·5	7
„	8	90	3	3	180	8	5	7	7
„	2·5	100	3	4	120	5	8	7	11
„	0·8	40	1	12	60	3	24	30	45
„	0·25	20	<2	5	40	5	14	25	48
M +	25	70	<2	45	140	<2	80	65	57
WS —	25	80	64	<2	160	140	3	80	90

B. Infectivity titrations of 8-hour first cycle fluids.

HA Inoculum	First cycle fluids		Types present at limiting dilution
	HA	ID_{50}	
25	160	$10^{7\cdot3}$	2 M +, 1 WS —, 1 MEL, 2 WSE
8	90	$10^{6\cdot5}$	2 WS —, 2 MEL
2·5	100	$10^{6\cdot0}$	5 M +, 1 MEL
0·8	40	$10^{7\cdot0}$	10 M +, 1 WSE
0·25	20	$10^{7\cdot3}$	12 M +, 1 MEL

* Sum of HA thus obtained in the two sera expressed as a percentage of the HA titre in saline.

The infectivity titrations are consistent with the results *in vitro*. The first three fluids show moderate degrees of incompleteness. In our experience fresh virus from embryos inoculated 2 days previously with very small doses of seed virus gives a ratio ID_{50}/HA with a mean value of $10^{5\cdot6}$, both titrations being made by our conventional techniques. In the present series the values for the ratio are $10^{5\cdot1}$, $10^{4\cdot5}$ and $10^{4\cdot0}$ for the three highest concentrations of inoculum, and within normal limits $10^{5\cdot4}$, $10^{6\cdot0}$ for the two lowest concentrations. Recombinants were readily obtained by allantoic titration to limiting infective dilutions of the first two fluids. The fluids from more dilute inocula contained a lower proportion of recombinants, and in the last two fluids only one recombinant

was obtained in two titrations of each. The types of virus shown in the Table are those obtained by inoculation of the two highest ten-fold dilutions to give rise to any positive fluids.

The excess of M + over WS — in the preparations receiving the smaller inocula, as shown by both *in vitro* and *in vivo* tests, is not explained.

Influence of the time at which the two viruses are added.

In these experiments one virus was added before the other, the first in a volume of 4·5 ml., the second at the appropriate time in ten times the final concentration in a volume of 0·5 ml. The inoculum was removed one hour after the last addition, and further treatment was along standard lines. Table 4A combines the *in vitro* results from two experiments, while 4B shows the recombinants recovered by a shorter method. To obtain MEL, the first cycle fluid was diluted 1 : 100 in anti-WS rabbit serum diluted in 1 : 25 and inoculated into six embryos.

TABLE 4.

Recombination in relation to the intervals between the addition of the two viruses.

A. In vitro *findings.*

	M + first				Together		WS — first			
Diluent	2 hrs.	1 hr.	30 min.	15 min.	0		15 min.	30 min.	1 hr.	2 hrs.
Saline	320	450	160	240	160	240	120	120	320	320
Anti-M	3	7	12	24	32	16	16	16	120	110
Anti-WS	240	160	32	24	3	16	10	32	8	8
p.c.	76	36	*28*	*20*	*22*	*13*	*22*	*33*	38	35

B. Presence of recombinants in first cycle fluids.

	M + first				Together		WS — first			
	2 hrs.	1 hr.	30 min.	15 min.	0		15 min.	30 min.	1 hr.	2 hrs.
Test for MEL with anti-WS serum in allantoic cavity	0/6[1]	6/6	4/5	3/4	5/6	3/4	1/5	2/3		
Test for WSE on C.A. with anti-M serum									2/2[2]	0/4

[1]Number of MEL fluids obtained/number of embryos inoculated.
[2]Number of embryos showing typical haemorrhagic lesions/number inoculated.

After two days the positive fluids were harvested and characterized. Those showing MEL serology and failure to develop the indicator state on heating to 52° C. were classed as MEL. Such fluids on retitration in eggs have always

given typical MEL strains, and the finding of MEL *in vitro* qualities has been accepted as adequate evidence of recombination.

To obtain WSE recombinants the method used was to dilute the first cycle fluid in anti-MEL serum to eliminate M + activity and inoculate on the chorioallantois (C.A.). If haemorrhagic lesions were produced in the embryos at 4 days and tissue emulsions gave haemagglutinin of WS serological character the presence of WSE was regarded as proven.

It will be seen from the results that recombination can occur when the difference in the times of adding is one hour or less. When the time is two hours there is no evidence of recombination by either method. There is marked evidence of interference in the sense that the virus given first opportunity predominates in the final yield.

The influence of time of emergence of virus from the cell on the proportion of recombinants.

In a standard experiment with equivalent amounts of M + and WS —, the procedure was modified by taking samples of fluid at 4 hours to obtain virus released early in the cycle. At 6½ hours the eggs were drained, washed once and 5 ml. of fresh Ringer added. After 2 further hours, during which virus liberated over this period was allowed to accumulate, this fluid was removed as a late sample for comparison with the early one. The results of two titrations of these fluids are shown in Table 5, the numbers of each type of fluid being given.

TABLE 5.

Virus types recovered from early and late fluids in a M +/WS — experiment.

	4 hrs.					6½–8½ hrs.				
Dilution	M +	WS —	MEL	WSE	Total	M +	WS —	MEL	WSE	Total
10^{-6}						2	4	1	2	9/25
10^{-6}						2	1	1		4/8
$10^{-5\cdot5}$		4		1	5/25	2	10	6	1	21/25
10^{-5}		5	1	2	10/12	2	3	2		8/9
10^{-5}	2	3	3	1	11/25					
10^{-4}	5	2	4		11/12	2	1			7/7
Total	7	14	8	4	$ID_{50}10^{5\cdot4}$	10	19	10	3	$ID_{50}10^{6\cdot0}$

Haemagglutinin content of early and late fluids.

	4 hrs.	6½-8½ hrs.	22½ hrs.
Saline	30	90	300
anti-M	2	10	50
anti-WS	<2	3	16

The main point to be derived from this experiment is the uniformity of composition of the two samples. Both fluids show typical double neutralization, the ratio between HA and infectivity titres is not significantly different and corresponds to 97–98 p.c. of incomplete virus, while finally the ratio of the four types is nearly identical. The combined percentages for the four types are

WS — 41 M + 24 MEL 25 WSE 10

In another experiment 4-hour and 22-hour fluids were compared. These contained a higher proportion of M + and WS — strains, but the ratio of recombinants to originals was similar in each. The 4-hour fluid gave 2 MEL from 13 fluids characterized, the 22-hour 7 MEL and 1 WSE from 34. There were 6 WS — to 5 M + in the 4-hour fluid, 17 WS — to 5 M + in the 22-hour fluid.

DISCUSSION.

The work reported in this paper is concerned with six main topics. The *first* is the technical convenience of the de-embryonated egg for work of this type. This is probably self-evident once the suitability of the preparation for virus multiplication is accepted. The *second* is the demonstration of the early liberation of virus into the fluid, traces being detectable at 3 hours, and relatively large amounts, including a proportion of recombinants, at 4 hours. This is largely due to the use of RDE, which allows virus to be recognized in the fluid immediately it appears on the free surface of the infected cell.

The *third* is the use of double neutralization, described in the first paper of this series as an index of the occurrence of recombination. This allows, in this particular system of M +/WS — at least, a much quicker method of determining the conditions under which recombination takes place. In view of the discussion below of the hypothesis of diploid virus, some reservations may be needed before one can regularly equate double neutralization with recombination.

The *fourth* is the demonstration of the necessity for the use as inoculum of a number of virus particles approximately equal to or greater than the number of susceptible cells available. This is in agreement with the *a priori* consideration that at least one particle of each type must enter the same cell if recombinants are to be produced in a single cycle of virus multiplication.

It would be expected that only a limited interval between the exposure of the cell to infection to the two types would be allowable. Following on similar work by Fraser (1953), the *fifth* point is concerned with the failure of recombination to occur if one virus precedes the other by an interval of 2 hours.

The *final* theme, and the only one calling for extended discussion, is the ratio of the virus types found when first cycle fluids are tested by titration at limiting infective dilutions.

The distribution of types in limit or near limit infective dilution fluids from titrations of first cycle fluids from de-embryonated egg preparations, given equivalent amounts of M + and WS — were fairly regular from experiment to experiment. Of a total of 178 typed fluids, the percentages of each type were

MEL 30 M + 24 WS — 39 WSE 7

In a similar experiment using the allantoic cavity 87 fluids gave

MEL 39 M + 22 WS — 33 WSE 6

but another of the same type gave a much reduced proportion of recombinants,

3 MEL and 1 WSE to 24 M + and 12 WS —.

The constant feature is in regard to the ratio of the two recombinants MEL to WSE, which ranges about 4–7 to 1.

Much less has been done with the opposite MEL/WSE system, but in this there is a consistent but less striking preponderance of WS — strains over M + strains.

The unbalanced ratio of MEL: WSE recombinants provides an interesting problem. It would be reasonable on any theory of reciprocal genetic interaction to find that whenever a MEL recombinant appeared there would also be one of WSE. The conditions of the experiment are such that the first cycle fluid can contain only virus liberated from first cycle multiplication largely in cells infected with more than one particle. One should expect on the simplest assumption a variable excess of the parents WS — and M +, but equal numbers of the recombinants MEL and WSE.

The *in vitro* phenomenon of double neutralization points strongly toward the existence of units carrying two different serological qualities, and perhaps equally two distinct genomes. Suppose we assume that all influenza virus particles are diploid in character and attempt to follow the implications of this hypothesis. From a double infection of a cell with two serologically different forms X and Y three types of virus would be liberated, XX, YY and XY. Limit dilutions would give 25 p.c. of XX and 25 p.c. of YY fluids. An XY unit inoculated into an embryo would, on infecting a cell, give rise to XX, YY and XY units which would go on to infect other cells, and generations would follow until a maximum concentration of virus was reached in the fluid. It is clear that the observed quality of such a fluid would depend on the result of a struggle for survival amongst XX, YY and XY. If XX made more rapid and effective contact with the cell surface than either of the others and the X components multiplied at least as readily as Y inside the cell, then the result would be that all XY units would give rise to XX type fluids after two days' multiplication in the egg.

When recombination has to be taken into account, the simplest postulate is to assume the existence of two independent linkage groups in each genome. A typical first cycle fluid will contain on this view equal numbers of each of

four genomes, MEL, M +, WS — and WSE, linked with one another at random to produce diploid virus particles. The various combinations can be sorted out as in Fig. 2 where, to avoid confusion, the genomes are listed as A, B, C and D.

AA	AB	AC	AD
BA	BB	BC	BD
CA	CB	CC	CD
DA	DB	DC	DD

Fig. 2. To illustrate the influence of dominance of one component in "diploid" virus particles, in determining the type of fluid eventually isolated. (See Text.)

If it should happen that AA has a clear survival advantage over any other combination and DD is incapable of surviving in competition with any other combination, then it will be clear that the product of doubly infected cells, as tested by titration at limit dilution, will be in the ratio 7A, 1D, 8B or C, i.e., 44 p.c. of A to 6 p.c. of D, or if recombination between B and C can give A, this will become 56 p.c. of A to 6 p.c. of D. The ratio is obviously close to that found experimentally for MEL to WSE in M +/WS — recombination experiments.

Following up this suggestion, equivalent mixtures were made of 100 HA units of fresh viruses of the four types in all possible pairs. The mixtures were diluted 10^{-6} and inoculated into 6 embryos each. The fluids were harvested at 2 days and characterized with the results shown.

MEL + M +	MEL + WSE	MEL + WS —	M + + WSE	M + + WS —	WS — + WSE
5 MEL 2 M +	6 MEL	6 MEL	6 M +	6 M +	4 WS — 2 WSE

Both MEL types clearly dominate both WS, and there is a less decisive advantage of MEL over M + and of WS — over WSE. Although there are still some minor anomalies, we feel that the hypothesis of diploid virus provides a satisfactory explanation for two phenomena, double neutralization of the haemagglutinin in first cycle fluids and the gross asymmetry of recombinant yields, for which there is no other simple interpretation. As has been pointed out above, most of the virus demonstrable as diploid by double neutralization must be incomplete, and it is only an assumption that the proportion of active virus has the same distribution of genotypes as the bulk of incomplete virus. A survey of our results shows nothing incompatible with such an assumption.

No attempt will be made to discuss the more strictly genetic aspects of the phenomena with which we are concerned. As far as the results reported here are concerned, they are compatible with the hypothesis that the characters concerned are distributed in two permanent linkage groups and that cross over has not yet been observed.

SUMMARY.

A modified technique for de-embryonation of fertile eggs is described which allows retention of the whole surface of the allantois for influenza virus growth.

With the standard technique involving treatment of the cells with receptor destroying enzyme RDE, haemagglutinin and newly produced infective virus are liberated earlier than in the normal allantoic cavity.

Recombination in the first infective cycle is only demonstrable if the dose of virus used is sufficient to ensure that a considerable proportion of cells will be infected with at least two virus particles.

When the interval between the addition of the two viruses to the system is one hour or less, recombination can be demonstrated. It is not shown when the interval is two hours.

With cells all initially infected and treated with RDE to minimize the likelihood of reinfection, virus liberation continues for a long period. The constitution of the virus liberated into the fluid at different periods is almost uniform in regard to the extent of anomalous neutralization, the relative apparent excess of one virus over the other, the degree of "incompleteness" of the virus and the types of virus isolated by allantoic inoculation at limiting infective dilutions.

The ratio of the recombinants MEL and WSE from M +/WS — experiments is approximately 4–7 : 1. Discussion of the significance of this ratio in the light of the existence of double neutralization leads to the conclusion that the viruses concerned normally have a diploid constitution.

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ACTION OF THE RECEPTOR DESTROYING ENZYME OF *V. CHOLERA* (RDE) IN GUINEA-PIGS

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The receptor destroying enzyme of *V. cholerae* (RDE) has been used extensively in the *in vitro* study of virus-red cell and virus-soluble inhibitor interaction. Use of the enzyme for *in vivo* investigation has been limited, due to the many impurities present in the crude preparation. Using a preparation purified by adsorption and elution from red cells, Burnet and Anderson (1947) found a small rise in the serum T. agglutinin following inoculation of RDE into guinea-pigs. The availability of high titre semi-purified preparations of RDE (Ada and French, 1950) made possible a re-examination of the *in vivo* action of this enzyme in guinea-pigs. The investigation was undertaken along two lines:

(a) the action of RDE on the electrophoretic mobility and agglutinability of the erythrocyte by viruses of the influenza group.

(b) the action of RDE on the serum inhibitor of "indicator influenza viruses" and on the titre of complement, cold agglutinins and T agglutinins.

A preliminary account of some aspects of these investigations has been given (French and Ada, 1950).

MATERIALS AND METHODS.

Guinea-pigs. Young healthy guinea-pigs of either sex 300 gm. to 500 gm. in weight were used. Unless mentioned, guinea-pigs were used for one experiment only and then discarded. Cardiac puncture was carried out under light ether anaesthesia using a 20-gauge needle. The majority of the animals stood this manipulation very well.

Receptor destroying enzyme. Purified enzyme, as prepared by the method of Ada and French (1950) was used. The titre of the enzyme was determined by allowing suitable dilutions in calcium acetate saline (pH 6·2) to react with a volume of washed 1 p.c. fowl erythrocytes for 1 hour at 37° C. The action of the enzyme was stopped by adding 1 drop (0·02 ml.) of saturated neutral sodium citrate solution followed by one volume containing 5 agglutinating doses of active MEL virus. The dilution showing the standard one plus degree of agglutination was taken as the end point.

Influenza viruses. The following virus strains were used in the form of infected allantoic fluid harvested from embryos inoculated at the age of 11 days and incubated for 40 hours at 35° C.

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Influenza A: egg adapted strains of MEL, PR8, WS, BEL and Swine.

Influenza B: egg adapted strains of LEE and MIL.

Newcastle disease virus of fowls (NDV): the Victorian strain described by Albiston and Gorrie (1942).

Indicator viruses T. MEL and H. LEE were prepared according to the method of Stone (1949).

Inhibitor titrations. Inhibitory titres were determined by using 5 agglutinating doses of indicator virus with 1 p.c. fowl erythrocytes. The inhibitor dilutions in normal saline and the indicator virus were left for 30 minutes at room temperature before addition of the erythrocytes which were allowed to sediment at room temperature. The end point was the standard degree of partial agglutination.

Complement titrations. Complement was titrated in calcium magnesium saline with 3 p.c. sheep erythrocytes sensitized with 5 MHD of haemolysin. Incubation was for 1 hour at 37° C., and the end point was the last tube to show complete lysis of the cells.

Cold agglutinin titrations. All sera were separated from the clot after incubation at 37° C. for 1 hour. Serial two fold dilutions of the serum to be tested were made in saline and a volume of 1 p.c. washed human group O cells was added to each dilution of serum. The mixtures were allowed to stand over-night in the refrigerator at 0 to 4° C., and the next morning examined for agglutination by gently shaking the tubes individually to make sure the agglutination was of the "firm type." The tubes were then transferred to the 37° C. incubator and the reaction read again after 2 hours. True cold agglutinin failed to react at the higher temperature.

T. agglutinin titrations. Human and guinea-pig erythrocytes were treated with RDE and titrations carried out using the method of Burnet and Anderson (1947).

Anti-RDE titrations. The technique described by Burnet (1949) was used.

Determination of electrophoretic mobility. Micro electrophoresis was carried out in the apparatus and manner described previously (Ada and Stone, 1950), the buffer used being M/15 sodium potassium phosphate, pH 7·35± 0·05 (Clark, 1920). Mixtures of cells of differing mobilities were estimated as follows. Due to the great difference in mobility of normal and RDE-treated cells (1·11 and 0·24 μ/sec./volt/cm. respectively), it was easy to recognize visually whether cells were of normal mobility or otherwise. In practice, the number of cells in focus in a selected area of the field of the microscope were counted, the current then turned on momentarily and the number of cells migrating slowly (or fast, whichever was the most convenient) counted. A total of at least 250 cells per sample was counted. As is explained later, there were often some cells of intermediate mobility present and the proportion of these could only be estimated very roughly.

EXPERIMENTAL.

Prior to the investigation of the *in vivo* action of RDE, the *in vitro* reaction of guinea-pig erythrocytes with RDE as followed by changes in electrophoretic mobility (E.M.) and agglutination with different strains of influenza virus was studied.

Normal guinea-pig blood was collected into citrated saline by cardiac puncture. The erythrocytes were collected and washed in physiological saline three times and finally packed by centrifuging for 20 minutes at 3,000 r.p.m. Dilutions of RDE were made in calcium acetate saline and to 0·25 ml. of each dilution 0·25 ml. of packed freshly washed guinea-pig erythrocytes was added. One ml. of calcium acetate saline was added, and after shaking, the mixtures

were placed in a 37° C. water bath for 30 minutes to allow the RDE to react. At the end of this time 2 drops (0·04 ml.) of saturated sodium citrate were added to each tube and the mixtures centrifuged. The cells were washed twice with citrated saline and then with a 1/10 dilution of an anti-RDE rabbit serum (Burnet, 1949) previously absorbed with RDE-treated red cells to remove the T agglutinins, followed by two more washings in normal saline. This step was found necessary to prevent the slow continued action of RDE adsorbed to the red cells. A portion of the RDE-treated washed erythrocytes was then prepared as a 1 p.c. suspension in saline, and this suspension added to tubes containing 5 agglutinating doses of each of the test viruses. The remainder of the cells was washed in the electrophoresis buffer and examined for their electrophoretic mobility.

TABLE 1.

In vitro *action of RDE on guinea-pig erythrocytes.*

Units of RDE per 0·25 ml. packed cells 30 min. at 37° C.	Electrophoretic mobility * μ/sec./volt/cm.	MEL (A)	PR8 (A)	NDV	WSE (A)	Swine (A)	LEE (B)	BEL (A)	MIL (B)
Nil	1·11	‡	‡	‡	‡	‡	‡	‡	‡
94	0·80	‡	‡	‡	‡	‡	‡	‡	‡
188	0·64	‡	‡	‡	‡	‡	‡	‡	‡
375	0·52	—	—	+	‡	‡	‡	‡	‡
750	0·41	—	—	—	—	+	‡	‡	‡
1,500	0·38	—	—	—	—	—	—	+	‡
3,000	0·31	—	—	—	—	—	—	—	‡
6,000	0·24	—	—	—	—	—	—	—	—

(A), (B), influenza virus type A and B. NDV = Newcastle disease virus; ‡ = complete agglutination of guinea-pig cells; + = partial agglutination; — = no agglutination of guinea-pig cells; * = determined in M/15 sodium potassium phosphate buffer pH 7·35.

Table 1 shows the results of such an experiment in which it can be seen that the progressive loss of agglutinability with the different viruses runs *pari passu* with a falling electrophoretic mobility of the erythrocytes, thus creating an "RDE gradient." This result acts as a basis against which the *in vivo* action of RDE can be compared.

Action of RDE on red cells in vivo.

In preliminary experiments, guinea-pigs were found to tolerate large doses of RDE injected intracardially in 1 ml. quantities. An experiment was set up to determine the *in vivo* course of action on the red cells.

Following inoculation of 100,000 units of RDE, small samples of blood were obtained at short intervals and washed as described previously (see above). The cells were then examined for their E.M. and agglutinability and the results are given in Table 2.

TABLE 2.

Agglutinability and E.M. of guinea-pig erythrocytes following inoculation of 100,000 units of RDE.

Time after inoculation	5 agglutinating doses of MEL (A)	PRS (A)	NDV	WSE (A)	Swine (A)	LEE (B)	BEL (A)	MIL (B)	Saline control	Electrophoretic mobility μ/sec./volt/cm.
30 min.	—	—	—	‡	‡	‡	‡	‡	—	0·50
60 min.	—	—	—	±	+	‡	‡	‡	—	0·42
142 min.	—	—	—	—	—	+	+	‡	—	0·36
202 min.	—	—	—	—	—	—	—	‡	—	0·30
262 min.	—	—	—	—	—	—	—	‡	—	0·26
23 hrs.	—	—	—	—	—	—	—	—	—	0·20
Control guinea-pig	‡	‡	‡	‡	‡	‡	‡	‡	—	1·10

It can be seen that the agglutination pattern is essentially the same as that obtained following *in vitro* treatment of red cells with RDE (see Table 1).

Influence of titre of inoculum on erythrocyte electrophoretic mobility and agglutinability. The time curves obtained following injection of varying amounts of RDE were next determined. Guinea-pigs were inoculated with 24,000, 12,000, 6,000 or 3,000 units and the electrophoretic mobility of the cells obtained at different intervals determined. The results are depicted in Fig. 1.

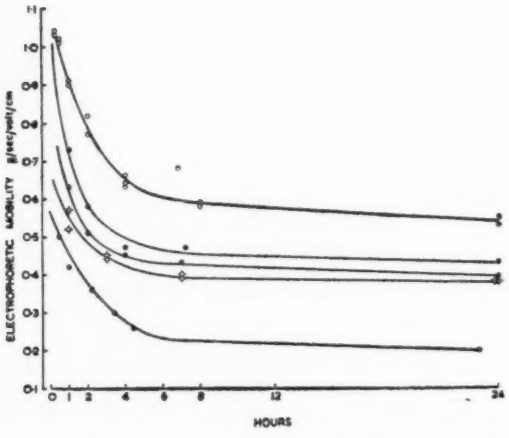

Fig. 1. Influence of the amount of RDE inoculated on the rate and extent of decrease in the electrophoretic mobility of guinea-pig erythrocytes.

Curves A, B, C, D and E represent the mobility values obtained following inoculation of 3,000, 6,000, 12,000, 24,000 and 100,000 units of RDE respectively. Curves A and E are constructed from values obtained using several guinea-pigs.

Injection of 100,000 units clearly gives the most rapid and extensive fall. Though it was desirable to reduce the E.M. of the red cells to a low value, it was also necessary in later experiments to economize with RDE. It was found that twenty-four hours after the injection of 50,000 units, the E.M. was reduced to a value of 0·33 (μ/sec./volt/cm.) and the injection of this amount was chosen as a standard procedure in subsequent experiments.

The agglutinability of the guinea-pig erythrocytes by the various viruses followed closely the fall in the electrophoretic mobility and was similar to the situation seen in Table 2. Where the mobility did not fall below 0·55 μ/sec./volt/cm. for example, the only viruses failing to agglutinate the erythrocytes were MEL, PR8 and NDV.

Return of erythrocyte mobility and agglutinability to normal. While examining cells from blood samples taken 4 or 5 days after inoculation of RDE, it was found that there were a few cells present whose electrophoretic mobility was noticeably greater than that of the bulk, and that the percentage of faster cells increased on subsequent days.

In a preliminary experiment three guinea-pigs were each given 50,000 units of RDE. Samples of blood were taken 1, 2, 4, 6, 8, 16 and 21 days after the inoculation. The electrophoretic mobility of erythrocytes from each sample was determined together with the percentage of cells whose mobility was greater than the value 24 hours after inoculation. The results showed that in each case only cells of normal mobility were present on the 21st day, although there was considerable variation in the rate at which cells of higher mobility appeared. The estimation of the proportion of cells of normal and slow mobility could not be regarded as accurate, due to the fact that a proportion of cells, very roughly estimated at 10 p.c., was of intermediate values. Cells showing such values appeared on the 4th or 5th day before any cells of normal mobility were present. By the 16th day, when there were no cells of very low mobility, some with intermediate values were present, such cells therefore being the last to disappear.

This experiment was open to the criticism that the frequent withdrawal of blood from the animals might interfere seriously with their normal haematological characteristics. To obviate this, an experiment employing 24 guinea-pigs was planned. Each was bled 3 ml. and the cells examined to establish normal values. The guinea-pigs were allowed to rest for a week in order to return to a normal haematological state. Each was then given 50,000 units of RDE and at intervals three animals were selected at random, bled by cardiac puncture, and their erythrocytes examined for electrophoretic mobility and agglutinability. The results are given in Table 3.

TABLE 3.

Return of agglutinability in vivo *of guinea-pig erythrocytes from RDE-treated animals.*

No. of days after RDE injection	p.c. of fast cells	MEL (A)	PR8 (A)	NDV	WSE (A)	Swine (A)	LEE (B)	BEL (A)	MIL (B)
0	100	‡	‡	‡	‡	‡	‡	‡	‡
1	0	—	—	—	—	—	—	—	‡
2	0	—	—	—	—	—	—	—	‡
4	4	±	±	±	+	+	+	‡	‡
6	20	+	+	+	‡	‡	‡	‡	‡
8	40	+	‡	‡	‡	‡	‡	‡	‡
16	95	‡	‡	‡	‡	‡	‡	‡	‡
21	100	‡	‡	‡	‡	‡	‡	‡	‡

The results are clearly similar to those obtained in the previous experiment with respect to the rate of appearance of cells of normal mobility, indicating that the frequent withdrawal of small amounts of blood did not materially alter the animals' response. It is also apparent that the agglutinability of the erythrocytes with the various viruses returns in an order which is the mirror image of that obtained by the action of RDE. A possibility existed that this effect might be due to RDE remaining in the vascular system, and to check this, two guinea-pigs were given 50,000 units of RDE, followed 24 hours later by anti-RDE guinea-pig serum to neutralize any remaining enzyme. The erythrocytes were examined 2, 4, 6 and 21 days after administration of RDE. The results were similar to those given in Table 3.

Evidence that the results shown in Table 3 were not due to simple replacement of the RDE-treated guinea-pig erythrocytes by normal cells is given in the following experiment.

A volume of washed guinea-pig erythrocytes was treated with an excess of RDE and after re-washing was made up in mixtures of varying percentages of normal and RDE-treated cells. These mixtures were then submitted to haem-agglutination by the usual viruses. The results given in Table 4 indicate that with the lower percentages of normal cells, there was a uniform degree of partial agglutination with all of the viruses.

TABLE 4.

Artificial mixtures of normal and RDE-treated guinea-pig erythrocytes agglutinated by viruses.

p.c. of normal cells	Agglutination by standard dose of								
	MEL	PR8	NDV	WSE	Swine	LEE	BEL	MIL	Saline
40	‡	‡	‡	‡	‡	‡	‡	‡	—
20	‡	‡	‡	‡	‡	‡	‡	‡	—
10	+	+	+	+	+	+	+	+	—
Nil	—	—	—	—	—	—	—	—	—

There appeared to be three possible explanations for the rapid disappearance of the slow cells in all these experiments, namely: (1) erythrocytes treated with receptor-destroying enzyme are removed at an abnormally fast rate and replaced at a similar rate by newly formed cells; (2) normal guinea-pig cells have an average life-span of less than twenty days; (3) there is no gross destruction of cells treated with receptor-destroying enzyme, the virus receptors being regenerated while the cells remain in circulation.

The agglutinability results obtained with artificial mixtures of fast and slow moving cells argue against the first possibility. Further evidence was obtained by estimating the extent of blood destruction following inoculation of RDE.

Reticulocyte counts on thin blood films vitally stained with Cresyl Blue were made on a number of normal guinea-pigs over several days. In these

animals the normal reticulocyte count (1,000 erythrocytes counted) was 0·1 to 0·5 p.c. with an average of 0·3 p.c. Two guinea-pigs, weighing about 450 gm. each, were bled by cardiac puncture 5 ml. and 8·5 ml. respectively, and their reticulocytes estimated every two days. In each case the reticulocyte count rose to just over 7 p.c. of the erythrocytes in 5 to 6 days.

Two guinea-pigs were given 50,000 units of RDE, and two the same quantity of RDE inactivated by heating in excess citrate at 60° C. for 30 minutes. The haemoglobin value and reticulocyte count were determined each day for eight days. Urine was collected from each of the guinea-pigs on several occasions and immediately examined for urobilinogen with Ehrlich's reagent. There was no significant difference between the guinea-pigs that were given the RDE and those given the inactivated RDE in any of these tests.

Despite the obvious sensitivity of guinea-pigs to blood loss as judged by reticulocyte response, there is no sign in either of the experiments of abnormally high blood destruction.

The choice between the second and third possibilities will be discussed later.

Transfusion of RDE-treated cells to normal guinea-pigs. As a further approach to the problem it was decided to transfuse a guinea-pig with RDE-treated cells and to follow, if possible, the fate of these transfused cells. A guinea-pig was given 50,000 units of RDE intracardially and 24 hours later the animal was bled out into citrate from the heart. The erythrocytes were treated with anti-RDE guinea-pig serum and then washed three times with saline. The packed, washed red cells were divided into 3 lots of 3 ml. and each was made up to 6 ml. with sterile pooled normal guinea-pig serum. Three normal guinea-pigs, approximately 500 gm. each, were anaesthetized and 6 ml. of blood removed from the heart and immediately replaced with the 6 ml. of RDE-treated cells in normal serum. Thirty minutes after this partial "replacement transfusion" the guinea-pigs were quite recovered and were again anaesthetized and 0·5 ml. of blood removed from each guinea-pig for examination of the erythrocytes. The average percentage of slow cells in this specimen was 13 p.c. Twenty-four hours later this figure had dropped to 8 p.c., and 72 hours after the transfusion only cells of normal mobility were present. No increase in urobilinogen was detected in urine specimens obtained from the guinea-pigs during this period.

Repeated injections of RDE. Guinea-pigs were given second and third injections of 50,000 units of RDE at monthly intervals. Examination of the erythrocytes following the second injection showed that the effect was considerably diminished, the mobility of the cells being reduced to the level expected with about one-tenth of the amount of RDE employed. The third injection of RDE, given one month after the second, failed to alter the electrophoretic mobility of the cells. Examination of the serum from these guinea-pigs showed

that this behaviour was due to the presence of anti-RDE in the serum. Following the second injection of RDE, the anti-RDE titre rose very sharply in the type of secondary response expected with soluble antigens.

Action of RDE on serum components in vivo.

All specimens of serum were stored at —10° C. until the end of each experiment. In this way variations such as that due to cells from different fowls (Anderson,. 1948) were eliminated. The results quoted below were largely obtained from the experiment described previously in which 24 guinea-pigs were given RDE and animals examined at intervals.

Titration of serum for influenza virus inhibitors. Table 5 shows the serum inhibitor titres of the guinea-pigs before and after inoculation of RDE. The figures were obtained by averaging the titres of the three sera for each day. It can be seen that the administration of RDE is followed by a sharp fall in the inhibitors for both indicator viruses. The inhibitor for H. LEE returns to normal before that for T. MEL.

TABLE 5.

The effect of RDE inoculation on the serum inhibitors of guinea-pigs.

Days after RDE inoculation	With T. Mel indicator virus		With H. Lee indicator virus	
	Titre before RDE	Titre after RDE	Titre before RDE	Titre after RDE
1	347	17	833	70
2	307	13	580	266
4	347	58	620	453
6	300	83	630	500
8	290	110	510	670
16	290	280	453	527
Control	363	400	933	1,133

Titration of serum T-agglutinin. Examination of all sera was carried out with the same batch of RD–H cells. Table 6 shows the average titres of the sera examined.

TABLE 6.

Effect of RDE inoculation on serum T-agglutinin in guinea-pig serum.

Days after RDE inoculation	Before RDE	After RDE
1	50	0
2	22	3
4	20	20
6	55	250
8	22	193
16	20	53
21	50	45
Control	42	45

Following injection of RDE, the T agglutinin titre fell precipitously and then rose to about 5 times the average normal level by the sixth day. No significant change in titre was observed by shaking the tubes and allowing them to re-settle at 37° C. When the titrations were carried out with RDE-treated guinea-pig cells, results similar to those found with RD–H cells were obtained.

Cold agglutinins. The sera had been separated from the clots without chilling the blood so that they were suitable for examination with human group O cells for cold agglutinin. All normal guinea-pig sera examined in this way were found to be negative at a dilution of 1/10. The results following RDE inoculation are given in Table 7. After treatment, a rise in titre was noted at the fourth day, reaching a maximum at the eighth day. At the 21st day after RDE treatment the serum still showed a cold agglutinin titre. Positive results obtained at 4° C. were abolished by shaking the tubes and re-incubating at 37° C.

TABLE 7.

Effect of RDE inoculation on cold agglutinin titre of guinea-pig sera.

Days after RDE inoculation	Before RDE	After RDE
1	<10	<10
2	<10	<10
4	<10	7
6	<10	60
8	<10	35
16	<10	11
21	<10	10
Control	<10	<10

When washing guinea-pig erythrocytes with the electrophoresis buffer, it was noticed that haemolysis frequently occurred with cells from RDE-treated animals, but never with erythrocytes from normal guinea-pigs. As the electrophoresis buffer is slightly hypotonic, it was decided to carry out saline fragility tests on guinea-pig erythrocytes after *in vivo* RDE treatment.

Fragility determinations (Parpart *et al.*, 1947) were carried out on normal guinea-pigs and guinea-pigs inoculated with RDE. Haemoglobin estimations were made with a photo-electric colorimeter and a distribution curve of erythrocyte fragility constructed (Hamburger, 1922; Bolton, 1949). Normal guinea-pigs uniformly gave a monomodal type of curve with a mediam at 0·40 p.c. NaCl. When guinea-pigs were given 50,000 units of RDE and their erythrocytes examined 24 hours later in the fragility test, the mediam was shifted to 0·5 p.c. but the curve was still monomodal. If, however, the guinea-pigs were examined six days after RDE treatment, then the curve was a bimodal one (Fig. 2). If the guinea-pig erythrocytes were examined 8 to 12 days after the

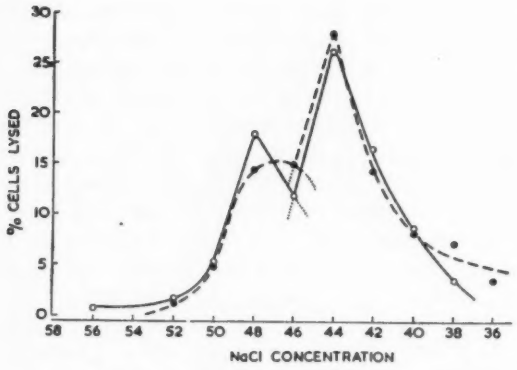

Fig. 2. Saline fragility curves of erythrocytes from two guinea-pigs six days after RDE inoculation.

administration of RDE the curve had a broad single mode instead of the sharply pointed type seen with normal animals. This was interpreted as due to the merging of the two modes into one.

DISCUSSION.

The work on the *in vitro* action of RDE on guinea-pig erythrocytes is comparable with the *in vitro* action of RDE on human red cells (Ada and Stone, 1950), the results being similar except for the different order in which the agglutinability of the various viruses is lost. As would be expected, the *in vivo* and *in vitro* agglutinability gradients are similar.

The injected RDE attacked the serum inhibitors of influenza virus haemagglutination and reduced them to a low level, but after 16 days the inhibitors returned to the normal level. This result indicates that the RDE has no permanent effect upon the cells or tissues responsible for the production of the serum mucoids. The first action on the T. agglutinins was to reduce the titre to a low level; this can be explained on the basis of adsorption of the serum T. agglutinins to the RDE-treated cells *in vivo*. The subsequent increase in titre between the sixth and eighth day above the original level confirms the experience of Burnet and Anderson (1947) and is probably due to over-correction by the guinea-pigs in response to the sudden removal of the T. agglutinin. Cold agglutinin was found to be absent from normal guinea-pig serum, but following RDE inoculation a titre was obtainable with a peak at the sixth day, and detectable agglutinin was still present 21 days after inoculation of RDE.

The finding that anti-RDE was produced by the guinea-pigs following injection of the enzyme seems at first sight to be at variance with the results obtained by Burnet (1949), who found that adjuvants had to be administered with RDE in his experiments to obtain an antibody response in rabbits. On closer inspection, however, it was seen that, using purified RDE, about 100 times as much enzyme was given in one injection as could be given in a course of injections with crude filtrates or even with red cell eluates. The antibody response without adjuvants in our case is, therefore, the result of the larger dose of enzyme administered.

The main interest in the work centred around the rapid appearance of red cells of normal mobility relatively soon after the mobility of all the circu-

lating erythrocytes had been lowered following the inoculation of RDE. Three explanations for this phenomenon were proposed and, on the basis of evidence obtained during the study, one was discarded. The remaining two were: that normal guinea-pig red cells have an average life span of less than twenty days *or* that there is no gross destruction of cells treated with RDE, the virus receptors being regenerated while the cells remain in circulation.

No information concerning the life span of normal guinea-pig red cells could be found in the literature. The figure of 20 days or less required in this case is substantially less than that found for any other mammal studied, although it is interesting to note that Neuberger and Niven (1951) have found marked variation in the red cell life span of individual rabbits. However, the experiment using mixtures of RDE-treated and normal guinea-pig erythrocytes, set out above and illustrated in Table 4, argues against the likelihood that the increasing agglutinability of the guinea-pig erythrocytes is due to new fully agglutinable cells being added to the circulation.

We are thus left with the alternative, namely, that the damage caused to the cell membrane by the RDE is repaired in the course of a few days. It is apparent from Table 3 that the receptors for the various viruses return in the reverse order from that in which they are removed by RDE. Thus agglutinability by MEL, PR8 and NDV is removed readily by small doses of RDE, but agglutinability for these viruses returns after the agglutinability for WSE Swine, LEE and BEL, i.e., the repair is the mirror image of the removal of the receptors. This evidence agrees well with the observed presence of a small proportion of cells of intermediate mobility during the repair process, implying that the normal mobility of each red cell is restored progressively over a period of some hours.

Repair of the erythrocyte surface *in vivo* implies that some constituents of the mammalian red-cell membrane may undergo rapid metabolism. Muir *et al.* (1951), have shown that there is an active turnover of cholesterol in the red cell which contrasts sharply with the metabolic inertia of the haemoglobin. This work and our observations are consistent with the view that there is a continuous reconstruction of the erythrocyte surface going on throughout the life of the cell. Hitherto it has been believed that such processes are confined to nucleated cells.

The observations on the production of cold agglutinins following the injection of RDE suggest that further work may possibly uncover a similar mechanism for the rising cold agglutinin titre observed in certain human cases of atypical pneumonia (Turner *et al.*, 1943). Similarly, the bi-modal fragility curves obtained (Fig. 2) recall similar curves described in certain human cases of familial acholuric jaundice and hypersplenism (Bolton, 1949). Further study of this aspect of RDE treatment of animals could possibly lead to some information concerning these not uncommon and distressing conditions.

SUMMARY.

Guinea-pigs were inoculated intracardially with purified preparations of the receptor destroying enzyme of *V. cholerae* (RDE) and the effect upon the erythrocytes and several serum components measured.

Within 24 hours after the inoculation of 100,000 units of RDE, the electrophoretic mobility (E.M.) of the erythrocytes had decreased from 1·10 to 0·20 μ/sec./volt/cm. At the same time, agglutinability for the various viruses of the influenza-Newcastle disease group was lost. By the 20th day after inoculation, however, only cells of normal E.M. and agglutinability were present in the circulation. Evidence is presented supporting the conclusion that the RDE-treated cells are not destroyed at an abnormal rate but that their surface can be repaired, implying that at least some constituents of the mammalian red cell may undergo rapid metabolism. Erythrocyte fragility was found to be temporarily increased by the RDE treatment.

The titre of serum haemagglutinin inhibitor and T-agglutinins were rapidly lowered following RDE inoculation. Cold agglutinins, absent in normal serum, were found, the peak value being reached six days after the inoculation. In all cases values returned to normal by about the 20th day.

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STUDIES ON THE SOLUBLE COMPLEMENT FIXING ANTIGENS OF INFLUENZA VIRUS

3. THE NATURE OF THE ANTIGENS

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In a recent paper, Hoyle (1952) has stated that the soluble C.F. antigen of influenza virus is a nucleoprotein. The claim is based on two points—that antigen preparations made either from a chorio allantoic membrane extract or from the virus elementary body by ether disintegration, formed insoluble complexes with lanthanum acetate and that digestion with crystalline ribonuclease for 48 hours at 37° C. decreased the complement fixation titre from 32 to 14. Until the antigen is obtained in a pure state neither test can be regarded as unambiguous. In particular it would be difficult to assert that the ribonuclease did not contain traces of a protease, particularly as it is known that trypsin rapidly inactivates the antigen.

Modern theories of living processes attach a key rôle to nucleic acid, and Hoyle's contention that the fundamental unit of the virus particle is the C.F. antigen makes it important that the nucleic acid content of the antigen be confirmed. In the present investigation, it has been considered essential to purify the antigen prior to analysis for nucleic acid. This has been accomplished in a two-stage process. Extracts of lungs from 18-day-old embryo chickens which had been inoculated two days previously with MEL virus were purified by differential centrifugation. The degree of purification was about ten-fold, the final solution containing particles of molecular weight about $1 \cdot 5 \times 10^6$ (Ada, Donnelley and Pye, 1952). These particles were considered to come from both infected and non-infected cells, and in order to obtain the C.F. antigen free from antigenically inactive components, it was precipitated from the mixture by complexing with specific antibody, using sera either from mice convalescing from influenza or from rabbits after a single inoculation (with adjuvants) of purified infected chicken lung extract (Ada, Perry and Pye, 1953). While the complexes formed could not be dissociated, they could be analysed for nucleic acid, and as serum proteins contain no nucleic acid, any such compound found must necessarily form part of the soluble antigen.

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MATERIALS AND METHODS.

Extracts. Extracts of infected embryo chicken lungs (ICLE) and normal embryo chicken lungs (NCLE) were prepared as described previously (Ada *et al.*, 1952, 1953). Prior to chemical analysis, an equal volume of cold 30 p.c. (w/v.) trichloracetic acid (T.C.A.) was added to the extract, the mixture let stand for 15 minutes at 0°C. and the precipitate removed by centrifugation. After washing with cold 10 p.c. T.C.A., the precipitate was suspended in the minimum amount of water, dialysed against water overnight, and dried from the frozen water *in vacuo*. Final drying was accomplished by evacuation in a desiccator over P_2O_5.

Antigen-antibody complexes. Complexes between ICLE and mouse convalescent or anti-ICLE rabbit serum were prepared as described previously (Ada *et al.*, 1953). After washing free from soluble protein, the complexes were dried from the frozen state *in vacuo*, the final stages being carried out over P_2O_5.

Complement fixation. The micro technique used has been described previously (Donnelley, 1951).

Chemical analysis. Initially, the lipid content was determined by extracting successively with boiling acetone, chloroform, alcohol, alcohol-ether and ether. Percentage lipid was obtained by comparison of dry weights before and after extraction. Phospholipid was precipitated from the lipid extract in the usual way (Bloor, 1929). The extracted residue was fractionated by the method of Schmidt and Thannhauser (1945) as modified by Davidson, Frazer and Hutchinson (1951) to determine the ribose nucleic acid phosphorus (RNA_P) and deoxypentose nucleic acid phosphorus (DNA_P).

During the investigation, an alternative method of extracting the lipid was adopted. Chloroform: methanol (2:1) was used (Folch and Lees, 1951). Extraction of the dried extract or complex was carried out for 2–4 hours at 0°C. After centrifugation at 2,000 *g* for 30 minutes at 0°C., the supernatant was carefully removed by Pasteur pipette and the residue washed with the solvent mixture. The proteolipid component was removed from the extract by diffusing against a supernatant aqueous layer (Folch and Lees, 1951). The lower chloroform layer was removed by centrifugation and the phospholipid content determined. The proteolipid was dissolved in methanol, transferred to a weighing bottle, the dry weight determined and analysed for phospholipid phosphorus. As proteolipid is approximately 50 p.c. lipid, total lipid content was regarded as the difference in weight between the original and the extracted material less half the proteolipid weight.

10–20 mg. of the extracted residue were then incubated at 37°C. overnight with 3 ml. N NaOH. After a clarifying centrifugation (if necessary), 0·15 ml. 10 N H_2SO_4 was added and the solution cooled to 0°C. 2 ml. of cold 30 p.c. T.C.A. were added and the mixture let stand at 0°C. for 15 minutes. After centrifugation at 2,000*g* for 30 minutes, the supernatant was removed and, if necessary, recentrifuged to remove traces of resuspended precipitate. The sediment was washed with 2–3 ml. of 10 p.c. T.C.A., centrifuged, and the washings added to the main supernatant. The residues were digested and the P content—representing the DNA_P—estimated. 3 ml. of the pooled supernatants (approx. 10 ml.) were used to estimate the inorganic P present—representing phosphoprotein P. The method used was that of Ernster, Zetterström and Lundberg (1950) with the slight modification that after shaking with benzene-butanol, the mixture was rapidly centrifuged. The lower aqueous phase was discarded and the small interfacial precipitate emulsified in the upper layer before a sample of the latter was taken. A further aliquot of the pooled supernatant was digested and estimated for total P. This value, minus that obtained for phosphoprotein P, was taken as the RNA_P. RNA and DNA values were obtained by multiplying the P values by the factor 10·3. Except in the case of phosphoprotein P, all phosphorus estimations were carried out according to Allen (1940).

EXPERIMENTAL.

Antigen-antibody complex analyses.

In Table 1 are given the nucleic acid contents of six complexes, two between ICLE and anti-ICLE rabbit serum and the other between ICLE and mouse convalescent serum. In the first four cases, the lipids were extracted using boiling acetone, etc. In the last two experiments, lipids were extracted using cold methanol-chloroform.

TABLE 1.

Nucleic acid content of complexes formed between C.F. antigens and immune sera.

Serum	RNA p.c.	DNA p.c.	RNA/DNA
Rabbit	1·25	0·31	4·0
Rabbit	3·4	0·69	4·9
Mouse	5·0	0·64	7·8
Mouse	4·2	0·37	11·3
Mouse	5·5	0·72	7·6
Mouse	2·7	0·32	8·5

It is evident from the analyses that both types of nucleic acid are present in the complex. The RNA/DNA ratios vary considerably. In the first experiment, the RNA content is considerably lower than in the other cases. In the first case, however, solubility studies at various pH values were carried out on the sample before analysis and the possibility exists that some of the nucleic acid may have been removed at the higher alkaline values. There is no suggestion that different methods of lipid extraction affect the amount of phosphorus left, which is estimated as nucleic acid.

A chromatographic analysis confirmed the presence of nucleic acid. Aliquots of two complexes (one with rabbit serum, one with mouse serum) were thrice extracted for one hour with boiling 10 p.c. (w/v) NaCl. The extract was shaken with chloroform until no further interfacial precipitation occurred. To the aqueous layer was added one drop of 0·1 N HCl and 2 vols. of ethanol and the resulting precipitate washed with ethanol and ether. It was then digested with N HCl at 100° C. for one hour and chromatographic analysis carried out in an isobutanol-HCl-water mixture (Smith and Markham, 1950). A sample of hydrolyzed yeast nucleic acid was run as a control. In both cases only four spots, as shown by ultraviolet absorption, were present and these corresponded closely in R.F. value to those given by the Y.N.A., thus confirming the presence of nucleic acid in the complexes.

Chemical relationship between C.F. antigen and normal tissue component.

It was of considerable interest to compare the properties of the antigen with those of preparations obtained by differential centrifugation from infected and normal embryo chick lungs. In Table 2 are given the values for different

fractions obtained from analyses of typical preparations from normal and infected lung extracts. Clearly, while the lipid values are quite similar, the nucleic acid contents differ. It is only with the nucleic acid values that the composition of the C.F. antigen itself can be compared, and in Table 3 are given the mean values obtained from analyses of six preparations of ICLE, six of NCLE, and the mean of the last five analyses of C.F. antigen-complexes given in Table 1. The first result in Table 1 is omitted due to the possibility, already mentioned, that the nucleic acid may have been extracted during solubility studies.

TABLE 2.

Composition of microsomal particles isolated from normal and infected lungs of embryo chicks.

Sample	Total lipid p.c.	Total phospholipid p.c.	Phosphoprotein phosphorus p.c.	RNA p.c.	DNA p.c.	RNA/DNA p.c.
NCLE*	24 (19·5–35)	5·0 (4·1–7·5)	0·013 (0·008–0·041)	9·1 (8·0–10·5)	0·11 (0·036–0·18)	83·0 (66–260)
ICLE†	21 (19·0–30·5)	5·3 (3·0–7·2)	0·028 (0·009–0·045)	6·5 (3·6–7·3)	1·0 (0·40–1·36)	6·5 (2·7–16)

* NCLE = Normal embryo chick lung extract.
† ICLE = Infected embryo chick lung extract.
Figures in brackets refer to range of values.

While there is a slight decrease in the amount of RNA in ICLE compared to that in NCLE, the striking difference lies in the increased amount of DNA associated with the ICLE.

TABLE 3.

Mean nucleic acid values of C.F. antigen-complexes, ICLE and NCLE preparations.

Sample	RNA p.c.	DNA p.c.	RNA/DNA
NCLE	9·6	0·11	87·
ICLE	6·15	0·83	7·4
C.F.A.-complex	4·2	0·54	7·8

Before proceeding further, it was decided to determine whether it was in fact DNA which was being estimated by the method used. A batch of ICLE was freeze dried and the lipid extracted with chloroform-methanol as usual. The dried residue was extracted thrice with 10 p.c. saline at 100° C. for one hour and the extracts pooled. In this way, about 70 p.c. of the phosphorus estimated in the original as nucleic acid was extracted. The extract was further deproteinized by shaking with chloroform, and nucleic acid precipitated from the aqueous layer by the addition of 2 vols. of ethanol, washed with ethanol, then ether, and dried. The sample was dissolved in 4 ml. of calcium magnesium saline and divided into two lots, A and B. A contained a total of 203 μg. of

P and B, 189 μg. of P. To A was added a few crystals of crystalline deoxyribonuclease and both were incubated at 37° C. for 5 hours. To each was then added 2 volumes of ethanol and the precipitates removed by centrifugation. The supernatants were estimated for phosphorus. The residues were fractionated for RNA and DNA by the standard method. The results are given in Table 4.

TABLE 4.

Effect of crystalline deoxyribonuclease on nucleic acid from ICLE. Percentage distribution of phosphorus in fractions.

Fractions	A With enzyme p.c.	B Without enzyme p.c.
Supernatant after nucleic acid precipitation	8·1	2·7
RNA	91·9	92·3
DNA	<0·2	5·0

The fraction estimated as DNA in the isolated nucleic acid is clearly completely susceptible to deoxyribonuclease (the RNA remaining unaffected) and indicates that the bulk of the component in the ICLE estimated as DNA is in fact DNA.

Relation between DNA content and the C.F. activity of ICLE.

The striking increase in the DNA content of the ICLE suggested that incorporation of DNA into the microsomal particle may in some way be connected with the appearance of antigenic activity. To test this, experiments were carried out in which extracts were made from lungs removed from the embryos at times varying from 16 to 72 hours after amniotic inoculation with virus.

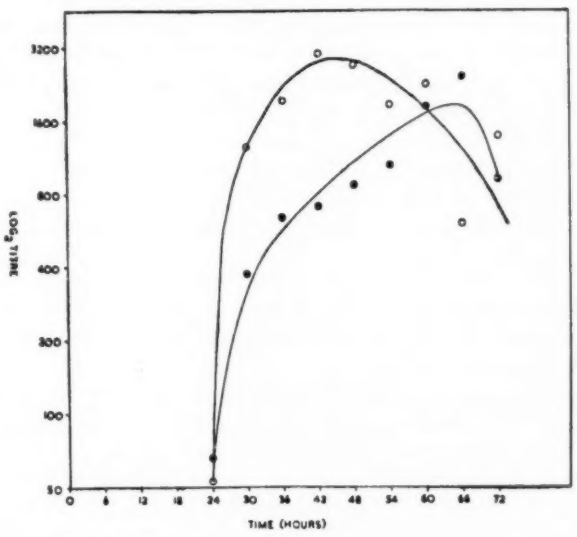

Fig. 1. Haemagglutinin and C.F. titres of extracts prepared from lungs of embryo chickens removed at intervals after amniotic inoculation of virus.

● = C.F. titres. O = Haemagglutinin titres.

A sighting experiment was carried out to find the most appropriate intervals to remove the lungs. Groups of six lungs were removed at six-hourly intervals up to 72 hours. Each lung pair was ground in 1 ml. saline and the haemagglutinin and the C.F. titres estimated. The mean values of the groups are plotted in Fig. 1. In both cases there is a sharp rise between 24 and 30 hours which flattens out and appears to fall by 72 hours.

TABLE 5.

Nucleic acid content and C.F. activity of purified preparations of ICLE obtained at varying time intervals after amniotic inoculation of virus.

Time (hours)	Dry wt. per lung pair	C.F. activity per mg. dry wt.	RNA p.c.	DNA p.c.	RNA/DNA
0	1·8 mg.	——	9·9	0·05	198
24	——	$12{\cdot}4 \times 10^3$	11·4	0·06	190
30	1·58 mg.	34×10^3	11·0	0·08	138
39	1·0 mg.	——	9·0	0·34	27
48	0·43 mg.	41×10^3	3·6	1·36	2·7
60	0·32 mg.	30×10^3	3·4	0·68	5·0
60 (control)	2·0 mg.	——	12·0	0·07	171

In Table 5 are presented the results obtained from an experiment in which lungs were removed from infected embryos at 24, 30, 39, 48 and 60 hours with controls from uninfected embryos at 0 and 60 hours. The first column gives the dry weight of purified material per lung pair and clearly shows the sharp decrease in the amount of microsomal component that can be obtained from the infected lung tissues in the later stages of the infection. Figures showing the C.F. activity per mg. dry weight of the purified preparations are given in the following column. Between the 30th and 60th hours, there are only slight differences agreeing with the general picture shown by the crude extract presented in Fig. 1. The values for RNA and DNA content and the RNA and DNA ratio follow. The decrease in RNA content is slightly more marked in this than in other experiments as is the correspondingly greater increase in DNA content. The controls at 0 and 60 hours show little variation in nucleic acid content, and a separate experiment in which uninfected lungs were removed from 14, 15, 16, 17 and 18-day-old embryo chickens showed relatively stable nucleic acid values.

It was of interest to see whether this increase in DNA content applied to any other fraction of the extract. The protein present in the supernatant following removal by high-speed centrifugation of the bulk of the C.F. activity was precipitated with T.C.A., dialysed, freeze dried and analysed as described under Methods. The results are presented in Table 6. In contrast to the microsomal fraction in Table 5, there is no marked decrease in the dry weight of supernatant protein per lung pair in the 24–48 hour extracts. There was an increase in the C.F. activity per mg. dry weight which largely occurred in the

24–30 hour period. A slight decrease in the RNA content was accompanied, however, by a marked increase in DNA content, the greatest increase occurring between the 42nd and 48th hour.

TABLE 6.

Nucleic acid content and C.F. activity of components in supernatant after removal by high speed centrifugation of bulk of C.F. antigen from extract.

Time (hours)	Dry wt. per lung pair	C.F. activity per mg. dry wt.	RNA p.c.	DNA p.c.	RNA/DNA
24	3·5 mg.	$0·22 \times 10^2$	1·21	0·01	121
30	2·3 mg.	$1·06 \times 10^2$	1·43	0·03	48
42	3·5 mg.	$1·3 \times 10^2$	0·88	0·04	22
48	3·5 mg.	$2·6 \times 10^2$	0·81	0·11	7·4

The main results of these experiments can be summarized as follows:

(1) There is no direct correlation between the appearance of C.F. activity and increase in the amount of DNA in the ICLE fraction. The major increase in C.F. activity occurs by the 30th hour whereas the main increase in DNA content is subsequent to this.

(2) Analysis of the supernatant protein between 24 and 48 hours after inoculation of virus showed a similar picture—the major increase in C.F. activity occurring by the 30th hour as compared with increased DNA content after this period. The C.F. activity per mg. dry weight of the supernatant protein was in each case, however, less than 10 p.c. of that in the microsomal fraction.

These results strongly suggested that the associated DNA was unrelated to the antigenic activity and the question arose whether it was an actual part of the antigen particle or could be regarded as a contaminant. It was thought that a histological approach might throw some light on the problem.

Smears were made of lungs removed at varying intervals after infection and stained with the Leishman stain. Although a quantitative approach was not made, the following impressions were gained. Up till 30 hours after infection, the majority of the cells appeared to be morphologically normal, occasional cells however showing damaged nuclei and many vacuoles in the cytoplasm. The proportion of damaged cells then increased until by the 48–60th hour after infection, few normal cells were present, most showing gross morphological changes. In addition, considerable numbers of leucocytes were also present and many of these showed signs of extensive damage.

DISCUSSION.

There is no doubt that the soluble C.F. antigens of influenza virus contain nucleic acid although the actual amounts are unknown. As the RNA : DNA ratios in the complexes are similar to those of the ICLE preparations, it is possible that the major components in the ICLE preparation are C.F. antigens.

In this case, the nucleic acid values found for ICLE (i.e., 6 p.c. RNA, 0·8 p.c. DNA) would approximate to the mean value of C.F. antigens prepared in this fashion. Davidson and Smellie (1952) have shown that the amount of RNA estimated by the technique used in this investigation is in excess of the true amount present, the error in the case of rat liver fractions being in the order of 25 p.c. The values given in this paper therefore must be regarded as maximal. It seems to be generally agreed that the values given by the technique used for DNA estimations are largely correct, Logan, Mannell and Rossiter (1952) having correlated the P' value with the ultraviolet absorption measurement. This is in agreement with the experiment given in the present paper in which the bulk (at least) of the DNA was found to be susceptible to deoxyribonuclease.

Evidence has been presented which indicates that the presence of DNA in the preparation is irrelevant to the C.F. activity. The experiments show that the increase in C.F. activity is not paralleled by the increase in DNA content, the two processes occurring at different times. Another fraction of the extract containing little activity shows a relatively similar increase in DNA.

In the early stages, around 30 hours after infection, there is a high titre of C.F. antigen and virus, and despite the absence of histological damage one can probably assume that the majority of the cells are infected and producing both agents. It is well known that very little gross change is visible histologically in allantoic cells infected six hours previously and at their peak of virus liberation. At 48, and still more at 60 hours, gross changes have occurred in the cells including great disorganization of many nuclei and a marked infiltration with inflammatory cells, mainly immature leucocytes, has occurred. DNA, perhaps partly depolymerized, from both these sources could well be responsible for contamination of both soluble and microsomal fractions.

Molecular considerations also tend to support such a conclusion. Assuming an average content of 1·0 p.c. DNA, this would allow a molecule of M.W. $1{\cdot}5 \times 10^4$ in a structure of mean particle weight $1{\cdot}5 \times 10^6$ (Ada *et al.*, 1952). This is considerably below reported values for isolated nucleic acid which are usually greater than 1×10^6. Suggestions have been made (e.g., Cohen, 1945) that in the native state and combined with protein, the value may be somewhat smaller, but little information is available on this point. If the M.W. is appreciably greater than $1{\cdot}5 \times 10^4$ there would be present less than one DNA molecule per antigen particle, indicating that the DNA was not an integral part of each C.F. antigen particle.

The evidence from the 24 to 30-hour preparations speaks strongly for the conclusion that, prior to the essentially irrelevant, later complications of nuclear damage, etc., the C.F. antigens are chemically similar to the normal microsome fraction prepared by differential centrifugation from uninfected cells. It is emphasized, however, that this conclusion may only apply to the situation present in the infected embryo chicken lung. As yet, there is little evidence to justify drawing analogies between this and other systems.

SUMMARY.

Antigen antibody complexes between semi-purified preparations of complement fixing (C.F.) antigens and either anti-C.F. antigens (semi-purified) rabbit serum or sera from mice convalescing from influenza have been prepared and analysed for nucleic acid content. Due to the absence of nucleic acid in γ globulin, any found was considered to be part of the C.F. antigens.

Analyses of five complexes gave the following mean result: ribose nucleic acid (RNA) = 4·2 p.c., deoxypentose nucleic acid (DNA) = 0·54 p.c., RNA/DNA = 7·8. The actual content of the C.F. antigens is unknown as the proportion of antigen in the complex could not be estimated. It is possible that the contents are similar to those of the semi-purified C.F. antigen preparations which had a mean of: RNA = 6·15 p.c., DNA = 0·83 p.c., RNA/DNA = 7·4.

These figures indicate a considerable increase in DNA content compared with those obtained from similar preparations from uninfected cells: RNA = 9·6 p.c., DNA = 0·11 p.c., RNA/DNA = 87. Experiments showed that the increase in DNA content of the particles occurred subsequently to the appearance of C.F. activity, and was not therefore directly associated with the antigenicity. Histological observations and molecular considerations supported the view that the DNA was not an integral part of each C.F. antigen particle, and it was suggested that the C.F. antigens were rather similar chemically to the microsomal fraction prepared by differential centrifugation from uninfected cells.

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THE EFFECT OF DESOXYRIBONUCLEIC ACID INHIBITORS UPON THE REPLICATION OF INFLUENZA VIRUS

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Recent work in this laboratory has been concerned with the analysis of variation in influenza viruses by the method of recombination. The evidence so obtained of genetic interaction between viruses seems only explicable if viruses possess a well-developed genetic mechanism broadly analogous to that in higher forms and responsible for the phenotypic qualities of the strains concerned (Burnet, Fraser and Lind, 1953). Since all genetic systems previously studied have contained desoxyribonucleic acid (DNA), it was of much interest to learn that influenza contains only ribonucleic acid (Ada and Perry, 1954). As influenza virus grows only in cells containing DNA, the absence of viral DNA does not preclude the necessity for DNA intervention in viral replication. In these experiments two widely used inhibitors of DNA, irradiation and nitrogen mustard (HN_2), were used to determine if host DNA is involved in the replication of influenza virus.

The morphological evidence for the effect of these materials on DNA has been recently reviewed by Hughes (1952).

Since ribonucleic acid (RNA) and protein are constituents of influenza virus, it was necessary to use concentrations of inhibitors which did not significantly affect these materials. Mitchell (1943) and several other workers since then (Holmes, 1947; Hevesy, 1949; Payne *et al.*, 1952; Stevens and Gray, 1953) have shown that doses of X-irradiation, which markedly inhibit the amount and turnover of DNA, have little effect on RNA or may even increase RNA and protein synthesis. Baron, Spiegelman and Quastler (1953) found no effect on protein synthesis, as evidenced by adaptive enzyme formation, in yeast subjected to 380,000 r. Lavik (1953) found that 400 r inhibited the incorporation of formate into adenine, guanine and thymine of DNA in the 14-day chick embryo by about 50 p.c. without affecting RNA adenine and guanine synthesis.

Bodenstein and Kondritzer (1948) reported that 5×10^{-4} M HN_2 inhibited DNA increase in Amblystoma embryos without effect upon RNA. Lowrance and Carter (1950) discovered that rabbits given HN_2 showed a marked depression in the specific activity of bone marrow DNA while RNA specific activity

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was unchanged. Stevens and Barron (1950), using C^{14} acetate and P^{32} found that concentrations of HN_2, which barely depressed respiration of rabbit bone marrow *in vitro*, produced a marked depression of DNA specific activity with minimal effects upon RNA or protein synthesis. This finding was used in the present experiments as an index to the concentration of HN_2 which would depress DNA turnover, but not RNA or protein.

The de-embryonated egg was particularly suitable for such a study as its system is relatively simple and solutions of known composition can be added or removed during the experiment.

Methods and Materials.

Fourteen to 16-day de-embryonated eggs were prepared as described by Burnet and Lind (1954).

For respiration studies, samples of the chorioallantoic membrane were removed, washed with saline, blotted, and 200 mg. placed in Warburg flasks containing 2·4 ml. modified Ringer phosphate buffer (Stevens, 1953) at pH 7·35. Duplicates from the same membrane were used for experimental and control samples. The HN_2 solution, prepared immediately before use as the hydrochloride, was added 1 hour before readings were begun. Readings were taken for a 2-hour period. The bath temperature was 37° C. and the gas phase was air. For the irradiation experiments, one half of the membrane was removed from the eggs and the eggs then irradiated using a 250 KVP machine at 15 ma. Other factors are given in the tables.

For experiments with virus, the eggs were de-embryonated and HN_2 in 5 ml. of Ringer's solution added to one group and Ringer alone to the control. These were rotated at 36° C. for 1 hour, then washed and 20 agglutinating doses of influenza A, strain MEL, added to all eggs in 5 ml. of Ringer containing penicillin (20 u/ml.), streptomycin (70 μg/ml.) and glucose (0·1 p.c.). After rotating 1 hour at 36° C., the fluid was decanted and 5 ml. of Ringer, containing 200 units of receptor-destroying enzyme (RDE) per ml. (Ada and French, 1950) was added. After 1 hour the eggs were washed twice and Ringer with antibiotics and glucose again added. In the experiments involving 27,000 r, a substrain of MEL, which has been found to have similar growth patterns, was used.

The 400 r irradiation was carried out 20 hours before the eggs were used. The heavy irradiation of 27,000 r was done after virus and RDE treatment had been carried out so as to avoid the possibility of affecting virus adsorption with this dosage.

Haemagglutinin titres were determined as described by Beveridge and Burnet (1946).

RESULTS.

Preliminary experiments revealed considerable individual variation in respiratory rates, but no consistent differences between 14, 15 and 16-day membranes.

Table 1 reveals a slight stimulation of respiration by 5×10^{-5} M HN_2 with beginning depression at 1×10^{-4} M, becoming more prominent at 2×10^{-4} M. The depression remained constant for at least 3 hours after the addition of HN_2. In contrast, Table 2 shows the increasing inhibition found

with 1×10^{-3} M HN_2, so that at 5 hours, respiration had almost ceased. From these results it was concluded that 1×10^{-4} M HN_2 would inhibit DNA synthesis without effect on RNA or protein.

Irradiation stimulated respiration at both 400 r and 27,000 r as shown in Table 1.

TABLE 1.

Effect of HN_2 and X-ray on oxygen consumption of 14–16 day chorioallantoic membrane.

HN_2 or X-ray	μLO_2/HR Control	Experimental	P.C. of control
0	89	89	100
	95	98	103
	94	90	96
	122	127	104
	85	85	100
HN_2 5×10^{-5} M	113	123	109
	87	97	111
	75	79	105
HN_2 1×10^{-4} M	75	72	96
	146	138	94
	153	144	94
	122	108	89
	106	88	83
HN_2 2×10^{-4} M	85	68	80
	113	95	84
	113	86	76
X-ray 400 r	38	83	224
	26	60	230
	39	73	188
X-ray 27,000 r	89	140	157

200 mg. tissue; 2·4 ml. buffer; pH 7·35; 0·15 ml. 15 p.c.; KOH in centre well; T = 37° C.; Air. Values are average rate between 1 hour and 3 hours after addition of HN_2 or X-ray.

TABLE 2.

Progressive inhibition of respiration by 1×10^{-3} M. HN_2.

Hours after HN_2 added	μLO_2/HR Control	Experimental	P.C. of control
2	86	55	64
	41	26	63
	72	48	67
3	86	33	38
	38	9	24
	70	27	39
5	78	14	18
	33	6	18
	64	7	11

Conditions as given in Table 1.

Tables 3, 4 and 5 demonstrate a complete lack of effect of HN_2 or X-ray upon either the early titres of virus or those reached after many hours.

In experiments using 1×10^{-3} M HN_2, no growth of virus occurred after 23 hours, irrespective of whether the HN_2 was added 1 hour before or 2 hours after the virus.

TABLE 3.

Effect of 1×10^{-4} M HN_2 on the replication of MEL (Influenza A).

	Controls	Treated
	320*	350
	320	320
	320	350
	450	350
	320	320
	320	320
	500	350
	400	320
	200	320
Mean	350	333

* Reciprocal of haemagglutinin titre at 20 hours.

TABLE 4.

Effect of 400 r on replication of MEL (Influenza A).

	Controls			Irradiated		
	4 hrs.	6 hrs.	23 hrs.	4 hrs.	6 hrs.	23 hrs.
	32*	300	800	16	80	300
	32	128	400	64	128	400
	32	96	500	40	128	800
	48	64	300	32	128	500
	32	192	800	32	96	300
	12	128	400	24	192	400
	48	128	500	48	192	500
	24	16	400	48	192	1,000
	48	128	300			
Mean	34	140	490	38	142	410

* Reciprocal of haemagglutinin titre. X-ray factors: 1 mm. Al. plus 0·5 mm. Cu filtration. HVL = 2·5 mm. Cu. 35 r/min.

TABLE 5.

Effect of 27,000 r on replication of MEL substrain.

Controls	Irradiated
325*	256
300	350
300	256

* Reciprocal of haemagglutinin titre at 18 hours.

X-ray factors: 1 mm. Al. filtration. HVL = 0·5 mm. Cu. 594 r/min.

DISCUSSION.

These data strongly suggest that DNA plays no rôle in the replication of influenza A virus. The finding of Blumenthal, Grieff, Chiga and Pinkerton (1953), that up to 1,000 r. (except for an aberrant result with 750 r) had little effect on the growth of PR8 in embryonate eggs would support this view.

In contrast to influenza, bacteriophage contains very large quantities of DNA with only traces of RNA. Herriot (1951) reported that concentrations of mustard gas, which prevented both division of *E. coli.* and synthesis of coli DNA, did not prevent multiplication of phage and that infected coli were capable of synthesizing DNA even in the presence of mustard gas. This would suggest that the mechanism of synthesis of phage DNA involves different pathways from that of coli DNA. Grieff, Chiga, Blumenthal and Pinkerton (1953) reported that irradiation was stimulatory to the growth of *Rickettsia mooseri.* Since Rickettsia contain DNA (Smith and Stoker, 1951), it would appear that their metabolism of this compound resembles the pathways of phage more than the pathways found in bacteria or higher cells.

The stimulatory effect on respiration noted with both 5×10^{-5} M HN_2 and X-rays is consistent with Barron's (1951) concept that labile thiol groups decrease respiratory processes and oxidation of these groups increases respiration.

Ackermann (1951) studied the respiration of the chorioallantoic membrane and reported much lower values than most of those reported here. While our first experiments all gave high values, the controls in the 400 r experiment and one of the 1×10^{-3} M HN_2 eggs gave values in the range of Ackermann's. It was noted that these membranes appeared thickened and less of the total membrane was required to supply 200 mg. Analysis of buffer and saline demonstrated them to be isotonic. Since subsequent experiments showed a correlation of the thicker type membrane with lower values, it was concluded that this variation was possibly referable to intrinsic differences in water content of different batches of eggs.

SUMMARY.

Concentrations of nitrogen mustard and amounts of X-ray which would markedly inhibit DNA synthesis had no effect upon the haemagglutinin titres of strain MEL of influenza A virus when grown in de-embryonated eggs.

It is concluded that DNA synthesis is unnecessary for the replication of influenza A virus.

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THE EFFECT OF PITUITARY POSTERIOR LOBE EXTRACTS ON THE BLOOD PRESSURE OF THE FOWL

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Pressor and oxytocic effects of posterior lobe pituitary extracts are assayed biologically by comparison with international standard powder, the former in terms of blood pressure increments in anaesthetized or spinal mammals and the latter generally, and up to a decade ago always, in terms of the contraction of guinea-pig uteri *in vitro*. Although there are precise directions in both the British Pharmacopoeia and in League of Nations Mems., 36 and 43, for the preparation of the glandular extracts for test, the precise conditions of the animal preparations are nowhere officially specified. This is of no practical consequence in the pressor assay, since all methods yield figures of comparable order; but the same is not true for the oxytocic assay. The British Pharmacopoeia *recommends* a saline of low magnesium content for use with the uterine strip (0·06 mg. p.c. Mg. compared with the mammalian serum content of 2·7 mg. p.c. Mg.). Using a uterine strip in saline of the lower Mg. content (or containing no Mg.), it is possible to distinguish between two fractions obtained by processing the simple extract. These are a pressor fraction (pitressin, post lobin V) with wholly pressor properties except for 3–5 p.c. contamination with oxytocic properties and an oxytocic fraction (pitocin, post lobin O) with wholly oxytocic properties except for 3–5 p.c. contamination with pressor properties. When the Mg. content of the saline is increased to 2·8 p.c. Mg., whole extracts, or mixtures of the two fractions in about equal proportion, record the same oxytocic titre as in a low Mg. bath, but high doses of pitressin, which exert no effect on the uterus in a low Mg. bath, now exhibit oxytocic properties (Fraser, 1939; Stewart, 1949).

Paton (1912) and Paton and Watson (1912) observed that injections of whole extract into the decapitated duck evoked a fall in blood pressure. Hogben and Schlapp (1924) showed that this also occurred in fowls and that the depression is not due to histamine or similar contaminants. Hogben (1925) further demonstrated that the depression is a specific property of pituitary extracts and that aqueous histamine-free extracts of other tissues (adrenal medulla excepted) have no effect on blood pressure of the bird. Gaddum (1928), working on the separated fractions obtained by Kamm *et al.* (1928), showed that the depressor response is elicited much more strongly by the oxytocic fraction (pitocin)

than the pressor fraction (pitressin). Coon (1939) demonstrated that the depressor effect could be used for the assay of oxytocic activity and his technique has been refined by Smith (1942), Smith and Vos (1943) and Thompson (1944). When dealing with unfractionated extracts, or with mixtures of fractions in approximately equal proportions, the fowl method yields the same figure for oxytocic activity as that obtained from the uterine strip in a saline of low Mg. content. This is because, although the fowl blood pressure depresses to pitressin, it is considerably less sensitive to it than to pitocin, and apparently any summation of effects is within the usual errors of assay. When, however, a mixture of pitressin and pitocin in a ratio of about 3:1 or higher is assayed, the depressor action of the pitressin is manifest and summates with the oxytocic evoked depression, so that the recorded oxytocic titre is higher. Thus the bird method offers a potential trap for the unwary assayist who records an oxytocic figure from this preparation without a check pressor assay: pitressin with only 3 p.c. oxytocic activity (measured on uterine strip in low Mg. bath) can yield a high oxytocic titre on the bird (*vide infra*). When the Mg. in the uterus bath saline is raised to the level of bird serum, 2·8 mg. p.c., the figures recorded by both methods with all mixtures are the same. *Inter alia* this implies that magnesium has a similar effect on smooth muscle response to pitressin at two anatomical locations.

Morash and Gibbs (1929) and Coon (1939) both noted that a series of pitocin doses, injected in close sequence into the fowl, led to a tolerant state when further doses of pitocin had less effect culminating in a state of complete unreactivity to pitocin. Pitressin injected into such tolerant preparations evoked a *rise* of pressure.

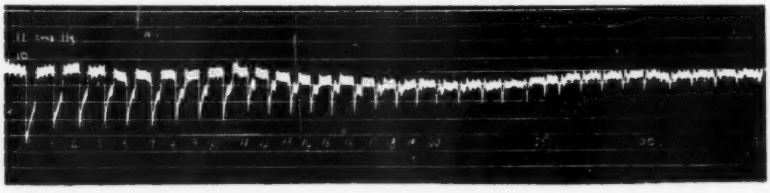

Fig. 1. Response to pitocin in a fowl with high blood pressure showing development of tolerance. Sodium phenobarbitone anaesthesia. All injections, 0·25 i.u. pitocin. 2–25 at 6-minute intervals, 25–35 at 3-minute intervals.

The present investigation was initiated to throw further light on the precise conditions predisposing to pressor and depressor responses to pitressin. Our object was to provide information relevant both to assay procedures and also to phyletic distribution of vasomoter responses to pituitary extracts (cf. Feakes *et al.*, 1950).

Materials and Methods.

Black Australorp roosters of heterogeneous stock averaging 3 kg. were anaesthetized with 200 mg./kg. soluble sodium phenobarbitone injected into a wing vein. The neck was dissected, the vagi and associated sympathetics severed, and the trachaea cannulated. The leg artery, vein and nerve were exposed as directed by Coon, the nerve severed, and the artery and vein cannulated. The rectal temperature was maintained at about 40° C. Artificial insufflation, when used, was at the rate of 40 strokes/minute.

RESULTS.

Response to pitocin.

In all preparations the response to a sufficient dose of pitocin was a precipitous fall in arterial pressure, a slower recovery, and sometimes a small secondary rise. The extent of the fall following a given dose is directly related to the base pressure of the preparation, the higher the pressure the greater being the fall. Tolerance develops quickly to large doses (above 1·0 i.u.), but however small the dose, the response to a series of injections in close succession is always less *at the end* (Fig. 1). Tolerance can be avoided by suitable spacing of doses, and, when tolerant, the sensitive state can be partially restored by a period of rest. Coon (1939) and Smith (1942) both record that complete tolerance can be induced by one large dose (25 i.u.). After a long series of injections, when the animal is showing tolerance to the depressor response, subsequent doses evoke secondary rises which may be equal to or greater than the fall (Fig. 2). This rise is sometimes larger than can be attributed to the 4 p.c. pressor contamination (*vide infra*).

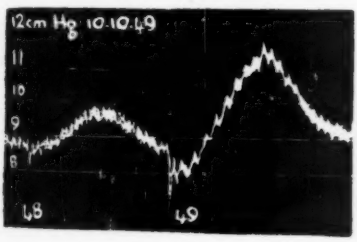

Fig. 2. Fowl under phenobarbitone anaesthesia.
Secondary rise exhibited to injections of pitocin alone after tolerance to depressor response is established. 48 = 0·1 i.u. 49 = 1·0 i.u.

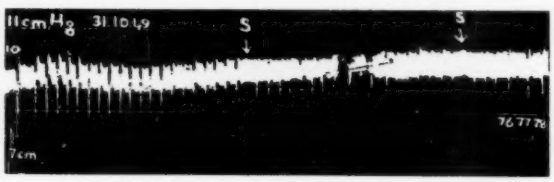

Fig. 3. Depressor response to pitressin and development of tolerance.
42 injections of 1·0 i.u. pitressin at 1-minute intervals. Last 3 doses, 76, 77, 78, are respectively 2·0, 3·0 and 4·0 i.u. "S" indicates injection of saline twice the volume of that used to wash in pitressin doses.

Injections of atropine, or of magnesium chloride, immediately prior to a dose of pitocin have no effect on the magnitude of the fall, this being substantially the same as that evoked by a preceding dose of the same concentration.

Response to pitressin.

Pitressin may evoke either a rise or a fall. The experimental data we have can be grouped conveniently as follows:

Response to pitressin when no other pituitary extract has been injected previously. Birds anaesthetized with phenobarbitone in ordinary (Coon) assay procedure usually have a *high base pressure* (10 cm. Hg. or more). Pitressin then evokes a sharp fall in pressure, the profile of the trace being indistinguishable from that evoked by a smaller dose of pitocin. There may be a small transitory secondary rise. The fall is not due to the pitocin contamination; this is shown by the inability of pitocin, in doses several times greater than 4 p.c. of the pitressin doses, to evoke an equivalent fall. Although we have not attempted to equate the depressor potency of pitressin and pitocin precisely in terms of international units, it may be said that in early injections, when tolerance complications are least, pitocin is about four to six times as potent as pitressin. Tolerance is exhibited (Fig. 3) to a series of doses in close succession, and as with pitocin, this may be avoided by suitable spacing of doses. Partial recovery from the tolerant state occurs after rest (Fig. 4). As with pitocin, the fall to pitressin is greater the higher the blood pressure at the time of injection. Table 1 demonstrates this relationship exhibited in a preparation in which the base-pressure was fluctuating in the absence of any apparent external stimulation.

TABLE 1.

Fowl under phenobarbitone. Effect of base pressure on depressor response to pitressin.

Time	Dose	Base pressure (mm. Hg.)	Response (mm. Hg.)
1626	1·0 i.u. pitressin	70	—20
1630	1·0 „ „	75	—20
1634	1·0 „ „	80	—20
1638	1·0 „ „	90	—22
1642	1·0 „ „	100	—24

TABLE 2.

Response to initial dose of pitressin (0·02–0·1 i.u.) at different base pressures.

Pressure (cms. Hg.)	Rise	Nil	Fall
11–11·9			**
10–10·9			*
9–9·9			
8–8·9	*		**
7–7·9	*		
6–6·9	*		
5–5·9	*		
4–4·9	**	*	

Each * represents one preparation.

A single experiment was made in an unanaesthetized bird. The blood pressure was high (12 cm. Hg.), a depression was evoked by pitressin and tolerance was developed to serial injections.

Pitressin injected into fresh preparations with a *low blood pressure* evokes a rise which may be preceded by a transitory fall (Fig. 5), and tolerance develops to this response also. All of the preparations listed in Table 2, which had base-pressures of less than 6 cm. Hg. and exhibited a rise to pitressin, were obtained accidentally and their low blood pressure may be ascribed to overdose of barbiturate, possibly also complicated by lengthy setting-up, citrate poisoning or surgical shock. (It may be noted that similar preparations which received initial doses of pitocin gave a normal depressor response.)

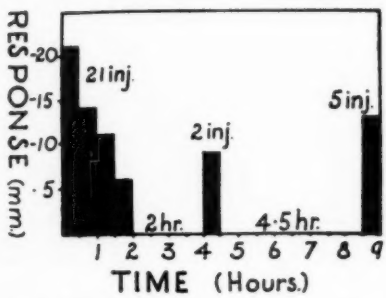

Fig. 4. Recovery from tolerance to pitressin (fall). Steps in histogram are arithmetic means of responses to serial doses of 0·4 i.u., 3 minutes apart, considered in blocks of half an hour.

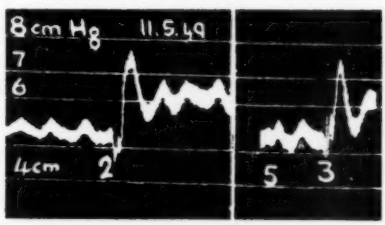

Fig. 5. Phenobarbitone anaesthesia. Rise to initial dose of pitressin in preparation with low blood pressure. 8 — saline injection. 2 and 3 = 0·04 i.u. pitressin.

In a first effort to decide whether this effect was conditioned by overdose of anaesthetic, or low blood pressure *per se*, two spinal preparations were set up according to the method of Paton (1912), blood flow to and from the head being prevented by a tourniquet around all the internal structures of the lower neck except the spinal cord. Instead of completely removing the head, as with Paton's "decapitated duck," the brain was destroyed by rotating a probe passed through the roof of the skull. Both preparations had a base-pressure of 4–5 cm. Hg., and both responded to pitressin by a rise in blood pressure and to pitocin by depression.

Attempts were then made to modify the blood pressure, without overdose of drugs or nerve section, to check the possibility of low blood pressure being a cause rather than a concomitant of the pressor response to pitressin. Preparations were set up in which blood could be quickly removed from and returned to the circulation; the left jugular vein was cannulated and connected to the stem of a separating funnel wetted with a small amount of heparin as anti-

coagulant. A dose of pitressin was injected and the depressor response noted, then about 150 ml. blood were swiftly drained into the separating funnel, lowering the blood pressure from about 9 cm. to about 4 cm. The pitressin dose was then repeated. In no case did this result in a reversal of the response. In general, it seemed that the depressor response was less at the lower pressure, but it was impossible to measure the response accurately due to the rapid recovery of the original base-pressure. We are hesitant about drawing firm conclusions from these experiments, but on their face value they seem to provide a *prima facie* case for investigating further whether the pitressin pressor response at low blood pressure is due to a concomitant of low blood pressure which takes time to manifest itself, as distinct from low blood pressure *per se*.

Response to pitressin of preparations tolerant to pitressin and pitocin.

Tolerant to pitocin. Preparations tolerant to pitocin, *at whatever base pressure,* exhibit a rise of blood pressure ("tolerance rise") when injected with pitressin; a depression to pitressin is never exhibited. Continued serial injections of pitressin lead to a refractory state, so that the preparation is then tolerant to pitressin as well as to pitocin.

TABLE 3.

Two preparations rendered tolerant to depressor response and now exhibiting tolerance rise to pitressin. Basal blood pressure elevated by intramuscular adrenalin in oil. Effect of subsequent injections of pitressin at two dose levels.

(A)	Time	Dose	Base pressure (in mm. Hg.)	Increment (in mm. Hg.)
	1047	0·2 i.u. pitressin	60	7
	1130	0·2 „ „	60	7
	1142	0·2 „ „	60	6
	1200	1·5 mg. adrenalin	—	—
	1230	0·2 i.u. pitressin	110	9
	1255	0·2 „ „	110	9
	1332	0·2 „ „	110	5
(B)				
	0205	1·0 i.u. pitressin	100	14
	0245	2·0 mg. adrenalin	—	—
	0322	1·0 i.u. pitressin	125	20

Tolerant to pitressin. Starting with a fresh preparation of base pressure 9 cm. Hg. or higher, initial doses of pitressin evoke a fall and a series of doses at 2–3 minutes interval leads to a tolerant state where little or no response is elicited. If the dose of pitressin is now increased, a depressor response may again be elicited (Fig. 3) *or* a rise of blood pressure ("tolerance rise"). Where the latter occurs it is itself subject to tolerance. We have been unable to determine precisely what determines which of these responses will occur, but what evidence we have implies that high base pressure at this stage favours a depressor response. In this connection we tried increasing the basal pressure

of preparations which had been rendered tolerant to the depressor response, and which were exhibiting a tolerance rise to pitressin. This was done by intramuscular injection of adrenalin in oil, pitressin being injected after a higher base pressure had become established. In no case did subsequent doses of pitressin evoke a fall, and in the only two (Table 3) in which a significant change occurred it was an increased rise. This effect may be, of course, quite independent of the blood pressure itself and due to synergistic action between the pitressin and adrenalin. We have no information about the effect of pitocin on a preparation tolerant to pitressin.

Effect of magnesium.

Prior intravenous injection of magnesium chloride produces a profound modification of the response to pitressin. In preparations exhibiting a fall to pitressin, increased circulating magnesium may increase the fall without modifying the second rise, or may decrease the secondary rise without increasing the fall, or both effects may be manifest (Table 4). In preparations tolerant to pitocin and exhibiting a tolerance rise to pitressin, magnesium injections cause subsequent pitressin injections to evoke a fall followed by a reduced rise or no rise at all (Table 5); this means that increased circulating magnesium can convert a tolerance rise to pitressin into a depressor response to pitressin.

TABLE 4.

Effect of magnesium on response to pitressin (see text for explanation).

(A)	Time	Dose	Response (in mm. Hg.) Fall	Rise
	1331	0·05 i.u. pitressin	5	3
	1343	2 ml. 2·1 p.c. $MgCl_2.6H_2O$	—	—
	1345	0·05 i.u. pitressin	13	4
	1347	0·05 „ „	12	2
	1408	0·05 „ „	2	3
(B)				
	1412	1·0 i.u. pitressin	32	3
	1419	2 ml. 2·1 p.c. $MgCl_2.6H_2O$	—	—
	1429	1·0 i.u. pitressin	31	—
	1431	1 ml. 2·1 p.c. $MgCl_2.6H_2O$	—	—
	1434	1·0 i.u. pitressin	30	—
	1452	1·0 „ „	28	6
	1500	1·0 „ „	30	10
	1508	1·0 „ „	32	11
	1525	1·0 „ „	30	14
(C)				
	1911	4·0 i.u. pitressin	6	14
	1915	2 ml. 2·1 p.c. $MgCl_2.6H_2O$	—	—
	1917	4·0 i.u. pitressin	14	8
	1923	4·0 „ „	7	15

TABLE 5.

Injections of pitressin and magnesium into a preparation tolerant to pitocin.

Preparation completely set up 12·00. 37 doses of pitocin given which evoked tolerance so that there was only a barely measurable response to 1·0 i.u. pitocin. Base pressure = 7 cm. Hg.

Injection	Time	Dose	Decrement (mm.)	followed by Increment (mm.)
1	2345	0·5 i.u. pitressin	0	20
2	0045	0·5 i.u. pitressin	Record defaced but approx. as in 1.	
3	0110	2 ml. of 2 p.c. $MgCl_2.6H_2O$ followed by 0·5 i.u. pitressin	4	32
4	0114	6 ml. of 2 p.c. $MgCl_2.6H_2O$ followed by 0·5 i.u. pitressin	10	33
5	0120	5 ml. of 4 p.c. $MgCl_2.6H_2O$ followed by 0·5 i.u. pitressin	8	20
6	0130	10 ml. of 4 p.c. $MgCl_2.6H_2O$ followed by 0·5 i.u. pitressin	10	0
7	0135	6·0 i.u. pitressin	13	6
8	0200	0·5 i.u. pitressin	8	12
9	0203	6·0 i.u. pitressin	15	12

Checked at termination of this series that preparation was still tolerant to pitocin.

When the dose is of the order of 3 mg./kg. body weight, injections of pitressin are affected to a decreasing degree for about 20 minutes, which is roughly the time taken for such a dose to disappear from circulation (Smith, 1942).

Response to mixtures of pitressin and pitocin.

For a full assessment of the mutual behaviour of pitressin and pitocin it would be necessary to have information about the response to mixtures in the following circumstances, (a) high blood pressure when animal is depressing to both pitressin and pitocin, (b) low blood pressure when preparation is exhibiting pressor responses to pitressin, (c) in tolerance rise to pitressin during pitocin tolerance, (d) in tolerance rise to pitressin after pitressin tolerance, (e) in the above cases after magnesium treatment. Actually we have only information on (a) and (c).

(a) The response of the fowl to small doses of pitocin contaminated with pitressin has been investigated by workers concerned with development of an assay method for oxytocic activity. They have shown that pitressin contamination has no effect on the fall evoked by a dose of pitocin unless the pressor: oxytocic ratio exceeds 2·5:1 (Coon, 1939) or 3·5:1 (Smith, 1942)[1]. Pitressin

[1]These statements are of course based on the doses usually injected in an assay, i.e., about 0·2 i.u. There are insufficient data to show whether they can be raised to the level of a generalization covering all dose ranges.

contamination in excess of these ratios results in a depressor response greater than would be accounted for by the pitocin constituent alone. The terms potentiation and synergism sometimes applied to this phenomenon may be misleading: certainly at this dose level it is something *less* than complete summation. Thus 0·2 i.u. pitressin would give a fall and so would 0·2 i.u. pitocin, but put in together as a mixture, or as whole unfractionated extract, they give a response only the same as evoked by 0·2 i.u. pitocin.

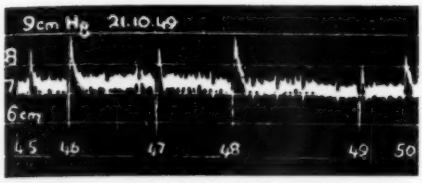

Fig. 6. Trace of data in Table 6B. The preparation had just begun its "tolerance rise" to pitressin (immediately previous dose of 1 i.u. pitressin evoked a pure rise of 6 mm.).

(c) In preparations exhibiting a tolerance rise to pitressin after attaining partial tolerance to pitocin, larger doses of pitocin will of course exhibit a depression (*vide supra*) and under these conditions mixtures of pitressin and pitocin greater than 2:1 evoke less fall (Fig. 6) than can be evoked by the pitocin component alone, and a subsequent rise equal to that evoked by the pitressin component alone (Table 6). This means that pitressin does not augment the pitocin depression when the preparation is partially tolerant, rather it implies that pitressin behaves as an independent pressor agent inhibiting the pitocin-depression and then exhibiting its own pressor action. Table 7 summarises the chief responses to pituitary extracts.

TABLE 6.

Effect of mixtures of pitressin and pitocin, at two dose levels, on preparations sufficiently tolerant to the depressor response to be exhibiting a rise to pitressin alone.

(A)	Time	Dose		Response (in mm. Hg.)	
		Pitressin	Pitocin	Fall	Rise
	1701	0·1 i.u.	—	—	6
	1716	—	0·05 i.u.	20	—
	1723	0·1 i.u.	0·05 i.u.	16	6
	1745	0·2 i.u.	0·05 i.u.	12	6
	1755	—	0·05 i.u.	19	—
	1808	0·2 i.u.	0·05 i.u.	14	8
	1848	—	0·05 i.u.	18	—
(B)					
	1723 (45)	2·0 i.u.	—	2	8
	1727 (46)	4·0 i.u.	1·0 i.u.	10	14
	1734 (47)	—	1·0 i.u.	15	8
	1740 (48)	6·0 i.u.	1·0 i.u.	11	14
	1751 (49)	—	1·0 i.u.	13	6
	1756 (50)	6·0 i.u.	—	2	9

Response to adrenaline and histamine.

As in the mammal, the response to intravenous commercial adrenaline (i.e., mixture of adrenalin and nor-adrenalin) is a swift rise. Sensitivity is high, 0·1 ml. of 1 in 100,000 evoking a rise of 22 mm., and 1·0 ml. of the same dilution a rise of 90 mm. There is no evidence of tolerance developing. No efforts were made to see whether a depressor response, comparable to that found in mammals, could be evoked by smaller doses.

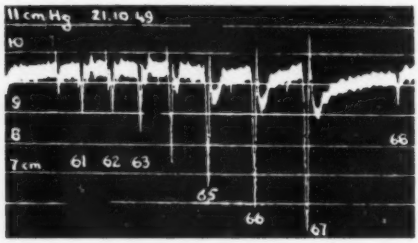

Fig. 7. Response to histamine. Continuous trace. A two-minute interval between doses 61 and 62.

61—0·001 mg. histamine acid phosphate.
62—0·001 " " " "
63—0·002 " " " "
64—0·005 " " " "
65—0·01 " " " "
66—0·02 " " " "
67—0·04 " " " "
68—0·001 " " " "

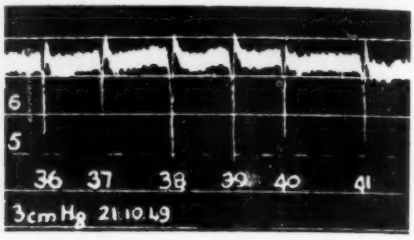

Fig. 8. Response to pitocin and histamine. Continuance trace; three-minute interval between 36 and 37.

	Histamine acid phosphate	Pitocin
36	—	1·0 i.u.
37	0·05 mg.	—
38	0·05 mg.	1·0 i.u.
39	0·05 mg.	1·0 i.u.
40	0·05 mg.	—
41	0·05 mg.	—

Injections of aqueous solutions of histamine acid phosphate result in a precipitous fall in blood pressure (Fig. 7). After large doses (0·1 mg. and more) there may be a transitory secondary rise. The depressor action of histamine and pitocin summate when the two substances are injected together (Fig. 8). When pitressin is evoking a fall followed by a small secondary rise, histamine injected at the same time increases the fall and reduces the secondary rise. We have no information about the action of histamine when pitressin is evoking a pure "tolerance rise."

DISCUSSION.

Two aspects of the fowl response to pituitary extract call for comment although we have little to contribute to a solution of the problems raised.

1. Pituitary extracts excite mammalian peripheral vessels in isolated preparations and in preparations treated with ergot; they also excite pulmonary vessel terminals which have no nerve connections (Dale, 1909). We can conclude, therefore, that neither a heart nor an intact nervous system is necessary for their action. Three responses to pituitary extract can be illustrated in

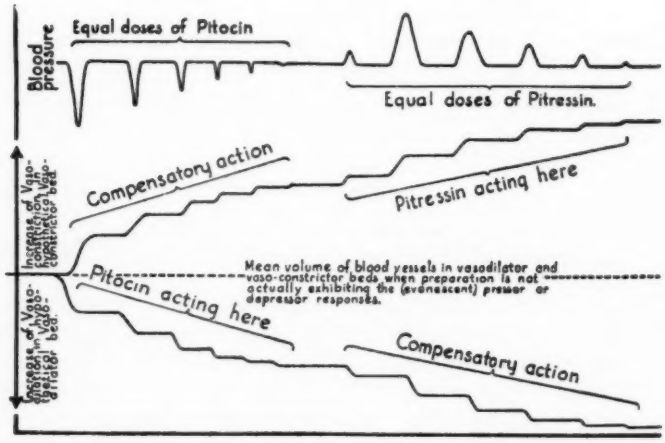
Blood pressure
Equal doses of Pitocin
Equal doses of Pitressin
Increase of Vaso-constriction Vaso-constrictor bed
Compensatory action
Pitressin acting here
Mean volume of blood vessels in vasodilator and vaso-constrictor beds when preparation is not actually exhibiting the (evanescent) pressor or depressor responses.
Increase of Vaso-dilatation Vaso-dilator bed
Pitocin acting here
Compensatory action

Fig. 9. Hypothetical scheme to account for development of tolerance to pitocin, development of tolerance rise to pitressin, and eventual development of tolerance to tolerance rise. At the conclusion of the two series of injections the preparation is tolerant to both pitocin and pitressin. It is implicit in this scheme that the vasodilator bed, having reached its limit to pitocin stimulation, can dilate farther as a compensation to further constriction, evoked by pitressin, in the vasoconstrictor bed (or, alternatively, an entirely separate vasodilator area is brought into play at this point).

birds, viz., pressor and depressor responses to pitressin, and depression to pitocin. Are these cardiac effects, or, if peripheral, are they manifest in different parts of the body, or any one part, or generally? Coon's (1939) work, showing that much larger doses of pituitary are needed to affect the heart directly than to evoke blood pressure changes, would seem to exclude direct cardiac involvement as a *major* component in the responses, and this is supported by our unpublished observation that pressor and depressor responses to pitressin and pitocin can be exhibited by a perfused isolated fowl leg. These perfusion experiments, taken together with Paton and Watson's observation that the splanchnic area enlarges during pituitary induced depressor responses, also suggest that most, if not all, peripheral tissues are responsive to pituitary stimulation, but this does not of course exclude the possibility that particular localized areas may be chiefly responsible for the full responses in the intact preparations. We have no direct evidence upon which to base further analysis.

2. Tolerance (tachyphyllaxis) is a well known concomitant of the pressor rise in mammals. The fowl exhibits tolerance to both depressor and pressor responses induced by pituitary. Dale (1909) has shown that in mammals no immune reactions are involved, so, if meanwhile we exclude these, five kinds of interpretation seem possible. These are merely set down here; there is no satisfying direct evidence permitting us to choose between them.

TABLE 7.

Fowl responses to pitressin and pitocin.

	Fresh preparation		Tolerant to	
	High b.p.	Low b.p.	Pitocin	Pitressin
Pitocin	—	—	+*	No data
Pitressin	—	+	+ at all b.p.s.	+ or — (probably according to b.p.)

— = depression. + = elevation.

* At least in some cases this is greater than can be accounted for on pitressin contamination.

(i) *Cardiac effects.* There have been no experiments to show whether or not cardiac effects are involved in bird tolerance phenomena, but the evidence cited above does not encourage the belief that they are.

(ii) *Exhaustion of substrates.* If pituitary extracts are not themselves excitant but act on substrates to produce the excitants, the gradual exhaustion of substrate could be the explanation of the gradual decreasing response to closely spaced serial injections, as has been shown to be the basis of the tachyphyllaxis evoked by serial injections of renin. Evidence from isolated muscle, admittedly indirect, does not favour this, but certainly does not exclude it.

(iii) *Alterations to blood ions* are known to result from pituitary injections, but there is no evidence about whether these changes affect the muscular response to subsequent injections.

(iv) *Saturation or inactivation of the receptor groups for pituitary excitants* on effectors might result from repetitive injection, but again there is no direct evidence.

(v) *Localized anatomical areas of the vascular bed with special properties* have been invoked by Larson (1938) to explain the tolerance phenomenon in mammals, and the thesis could be extended to explain *some* of the more complicated tolerance phenomena in birds. The first assumption required is that some area, e.g., spleen, changes its state of contraction under the influence of pituitary stimulation and remains in the changed state for long periods. Thus, after injection of pitocin, there is vasodilatation in the special area with consequent lowering of systemic blood pressure, the rapid recovery of recorded pressure being due to compensatory vasoconstriction elsewhere. Further doses increase the dilatation in the specialized area, but by smaller and smaller increments as the area approaches maximum dilatation. This hypothesis can account for the known effects of time and dose on the development of tolerance, and, by the further postulation of an analogous "vasoconstrictor area" sensitive to pitressin, can cover the emergence of the "tolerance rise." Some possible implications of this hypothesis are shown in Fig. 9. Larson advanced this view of mammalian tolerance after noting that the spleen does in fact behave in the manner required and the oral mucous membrane is also known to behave similarly. There is no experimental evidence for, or against, this proposition in birds.

SUMMARY.

In fowls anaesthetized with phenobarbitone:

Pitocin is always depressor (with the possible exceptions noted on p. 195).

Pitressin is depressor at high basal pressures in fresh preparations.

Pitressin is pressor at low basal pressures in fresh preparations, and in preparations tolerant to pitocin at any pressure.

Increased doses of pitressin injected into fowls tolerant to a given (depressive) dose of pitressin may evoke a rise or fall. Which response occurs probably, but not certainly, depends on the basal blood pressure at that time.

All responses to pituitary are subject to tolerance.

Magnesium injections prior to pitressin injections can in various circumstances augment a depression, eliminate a rise of pressure, or convert an anticipated rise into a fall.

Increased circulatory Mg. has no effect on responses to pitocin.

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PHARMACOLOGICAL STUDIES OF GRASSES OBTAINED FROM A PROPERTY WHERE "TALL FESCUE LAMENESS" IN CATTLE OCCURS

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In discussion with Pulsford (1949) it was thought worthwhile to investigate the pharmacological effect of a grass—tall Fescue (*Festuca arundinacea*)—which had been reported in New Zealand as associated with a peripheral vasoconstriction and dry gangrene in cattle grazing upon it. We were readily able to show that extract of the grass was pressor to the blood pressure, and it appeared likely that, as suggested from New Zealand (Cunningham, 1949), the effect might not be due to an ergotamine derivative. In the process of determining this, we investigated the effect of other grasses and found that the most active was *Lolium perenne* (Rye), which does not produce lesions in cattle when grazing on it. In this paper we have demonstrated and investigated the mechanism of this pressor response.

METHODS.

Grass extract.

Grass was cut up finely, ground with silica and boiled for 45 minutes in 0·6 p.c. acid saline. The mixture was cooled and neutralized with alkali.

Cat blood pressure.

Cats were anaesthetized with ether and chloralose, the carotid pressure recorded and the vagi cut. Injections were made into the femoral vein.

Adrenalectomy.

In certain of the animals the adrenal glands were removed.

Isolated uteri.

Rat. The isolated uterus of the virgin rat was suspended in a bath of Tyrode (7 ml.) through which oxygen was bubbled.

Cat. The uterus of the cat was also isolated and suspended in a bath of Tyrode for assay of extract.

[1]This work was aided by a grant from the National Health and Medical Research Council.

EXPERIMENTAL.

The effect of extract on the blood pressure of the cat.

Following the intravenous injection of extracts of *Festuca arundinacea* (Fescue) and *Lolium perenne* (Rye) there is a pressor response. This is shown in Fig. 1, where at F a delayed rise of B.P. is shown to 360 mg. Fescue extract, and at R an immediate rise followed by a greater delayed rise is shown to the injection of 220 mg. of Rye extract. It is noted that the response to adrenalin (10 μg.) at A_{10} is, if anything, a little greater following the injection of Fescue extract and probably due to the initially slightly lower B.P. Were the extract to contain an ergot-like material one would have expected the response to adrenalin to have been reduced. Following the second injection of extract (R) the response to adrenalin was further slightly increased, and this again was consistent with the response following from a lower initial B.P. The delay in the rise of B.P. could conceivably indicate that the pressor effect was not due to a direct action on the heart or immediate vasoconstriction. The delay is consistent with the development of a secondary output of a pressor substance, an aspect which is investigated later. Further, it is noteworthy that there was an initial immediate pressor response of lesser degree to the main rise following the injection of Rye extract, the heart beat faster, and the shape of the curve was very like the immediately preceding response to 2 μg. adrenalin (A_2). If this rise were due to adrenalin it would indicate a content of 9 μg. adrenalin per gm. of fresh grass. Further assay of this same sample indicated, however, a more active effect on the uterus (see below) of the cat than is the case with adrenalin in this concentration. This might indicate that the immediate pressor response is either not due to adrenalin, or that other substances present vary the response of the blood pressure and the uterus. The second explanation is not without merit in view of the recent finding that nettle contains acetylcholine and histamine (Emmelin and Feldberg, 1947). Again, as has been remarked upon earlier, the shape of the pressor rise is similar to that of adrenalin (possibly similar in mode of destruction) and the heart rate increased. The effect could not be due to potassium since the action would then be hypotensive.

In view of the greater pressor activity of *Lolium perenne* (Rye) while this grass was taken from an area on which cattle grazed without developing necrotic lesions it is indicated that either this pressor principle is not responsible for the disease in cattle or that there is some other substance in *Festuca arundinacea* which in some way potentiates the effect following ingestion in cattle, or that this grass lacks a substance present in other grass which is protective.

The uterus of the virgin rat.

The response of the uterus of the virgin rat to grass extract was investigated to compare it with ergotamine, a pressor substance which has been found to be causal in another disease caused through grass (infected with *Claviceps pur-*

purea). These findings are shown in Fig. 2, where at E is shown the inhibitor response to 1 μg. ergometrine, which is a little greater than the depressor effect produced by 35 mg. "control"[2] grass, C_{35}. The response to later doses is reduced as is shown by the response to 70 mg. "control" at C_{70}, which is a little less active than 1 μg. ergometrine. However, in view of the lack of effect of grass extract on the response to adrenalin (*vide infra*), it would appear that the extract cannot contain an ergot-like derivative. At H is shown the response to 40 μg. histamine, also depressor.

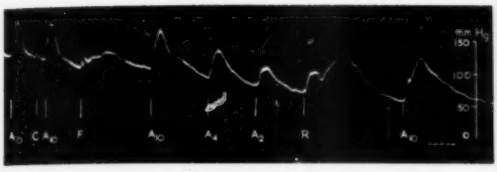

Fig. 1.

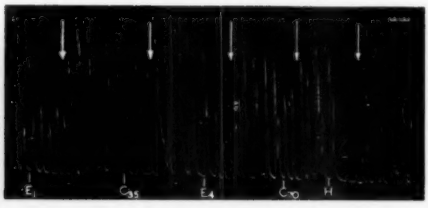

Fig. 2.

Fig. 1. Carotid blood pressure response of the intact cat. Ether and chloralose anaesthesia, vagi cut. At F, response to extract of 360 mg. Fescue injected intravenously; at R, response to 220 mg. Rye extract; at A_2, A_4, A_{10}, response to 2, 4, and 10 μg. adrenalin. Time in minutes.

Fig. 2. Response of the isolated uterus of the virgin rat. At E_1, 1 μg. ergometrine injected into bath; at E_4, 4 μg. injected. At C_{35}, 35 mg. control Rye extract injected into bath; at C_{70}, 70 mg. injected. At H_{40}, 40 μg. histamine injected. At the arrows the bath was washed out. Time in minutes. Further details in text.

The effect of extract on the uterus of the cat.

"Fescue" extract produces contraction of the uterus of the cat, and this is shown in Fig. 3 where, at F_{18}, 180 mg. "Fescue" extract produced a marked contraction. On the contrary "Rye" (*Lolium perenne*) in small doses just produced relaxation of the uterus as adrenalin does. This is shown at C_2, where 20 mg. produced inhibition a little less than that produced by 1 μg. adrenalin. This indicated a pressor content of equivalent approximately to 40 μg. adrenalin per gm. At C_7 the response to a larger dose (70 mg. "Rye") showed in addition a contraction following the initial depression. This rise was equal to the rise produced by 180 mg. Fescue, i.e., Rye extract is therefore two and a half times as active as Fescue extract as a stimulant to muscle of the uterus. If one compares the pressor response of these two extracts (tested on the same day, see Section 1), it is noted that 220 mg. of Rye extract produced a rise of pressure of 60 mm. Hg. while the response to 360 mg. Fescue was only 15 mm. Hg. It would appear therefore that, compared with Fescue, the pressor response to

[2]Control grass was a mixture containing mainly perennial Rye (*Lolium perenne*) which had replaced the Fescue on an area where cattle developed the disease while it contained Fescue.

Rye grass is greater in proportion to its stimulant action on uterus of the cat. This does not, however, necessarily indicate that the delayed pressor response to Rye is due to a different substance from that of Fescue since Rye contains, in addition, an immediate pressor substance which will add to the vascular response. Further, Rye produces a relaxation effect initially on the uterus of the cat which will reduce its stimulant action. It is to be noted that the response of the uterus of the cat to adrenalin was quite unimpaired following the injection of grass extract into the bath, indicating that there is no ergot-like derivative present. This is shown at A, A, A, where each response is that to 1 μg. adrenalin.

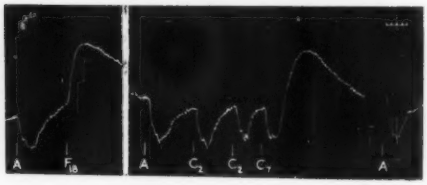

Fig. 3.

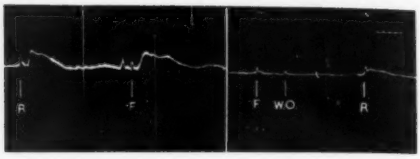

Fig. 4.

Fig. 3. Response of the isolated uterus of the cat. At F_{18}, response to 180 mg. Fescue extract; at C_2, C_7, response to 20 and 70 mg. control Rye extract. At A, response to 1 μg. adrenalin. Time in minutes. Further details in text.

Fig. 4. Response of the blood pressure of the cat. Ether and chloralose anaesthesia, vagi cut. Left-hand panel: at R response to 360 gm. Rye extract, at F to 360 gm. Fescue extract. Between the panels: bilateral adrenalectomy performed. Right-hand panel: at F response to 360 mg. Fescue extract; at WO response to 3 ml. saline and wash out of cannula; at R response to 360 mg. Rye extract. B.P. mm. Hg. Time in minutes. Further details in text.

Mediation of pressor response through the adrenal.

We have already indicated the possibility that the pressor response to grass extract (owing to the delay) may be mediated through a secondary mechanism. We were able to show that, in the absence of the adrenal glands, the pressor response to the intravenous injection of grass extract is negligible. This is shown in Fig. 4, where at F (left-hand panel) a delayed pressor response is noted which followed the injection of 360 mg. Fescue. A similar response was obtained to 360 mg. Rye (R). Between the panels the adrenal glands were removed and the response to 360 mg. Fescue (F) was abolished and that to Rye (R) (right-hand panel) was negligible. Evidently, therefore, the pressor response to grass extract is a secondary one due to liberation of adrenalin, and probably nor-adrenalin, from the adrenal. After adrenalectomy the response to adrenalin is ordinarily increased, and it might be that the slight pressor response obtained with Rye (though notably absent with Fescue) might be due to the substance producing the slight immediate rise of blood pressure in the intact cat described in section 1, which is "adrenalin-like."

DISCUSSION.

Our attempt to find a pharmacologically active substance to account for the necrotic lesion of tall Fescue lameness of cattle was unsuccessful. On the other hand we were able to demonstrate the presence of a potent vaso-pressor substance in the grass on which cattle suffering from that condition graze. This activity was even more pronounced in other grass, particularly Rye. It is true that these other grasses are more readily extractable, being not so hard and firm as Fescue. However, our boiling with acid saline for three-quarters of an hour might be regarded as a fairly severe procedure nullifying moderate differences in texture as inhibiting complete extraction. Further, if this destroyed activity it would apply to both extracts, and yet the more fragile Rye contained greater pressor activity.

The extract of grass is vaso-pressor in the intact cat, stimulant to the uterus of the cat, and rhythm inhibiting to the uterus of the virgin rat. The response is not due to an ergotamine type of substance as the response of the uterus of the rat diminishes with repeated doses, while that to ergotamine of equal intensity is little altered. Again the treatment of the cat uterus with extract has no effect on the amplitude of the response to adrenalin, which should be reduced were the extract to contain ergot-like activity.

We were then able to show that the pressor effect (quite delayed) following the injection of extract into the intact cat was mediated largely through the adrenal. This was shown by the response following injection being largely abolished after adrenalectomy.

SUMMARY.

Fescue grass, obtained from a property where "tall Fescue lameness" had occurred previously, was found to contain a pressor principle which acted in the intact animal by stimulating the adrenal gland. Rye grass also possessed this activity in even greater degree.

Both grasses were stimulating to the uterus of the cat and inhibitory to the uterus of the rat.

Neither grass affected the response of the intact cat blood pressure or the isolated uterus of the cat to adrenalin.

Rye grass extract had, in addition, a slight immediate pressor effect on the blood pressure.

The significance of these findings in relation to tall Fescue lameness is discussed.

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THE ROLE OF TISSUE ANTIGENS IN HAEMOLYTIC DISEASE OF THE NEWBORN

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It is well recognized that haemolytic disease of the newborn is nearly always associated with maternal immunization by foetal Rh antigens. The resulting Rh antibodies pass across the placenta to unite with and destroy foetal red blood cells and the anaemia so produced leads to tissue anoxia. This simple explanation of the pathogenesis of the disease may not, however, be the complete explanation. Death not infrequently occurs in the absence of anaemia and localized tissue changes are sometimes observed, suggesting specific damage to certain areas rather than generalized anoxia.

Various theories have been advanced to explain the pathogenesis of the disease. It has been suggested (Darrow and Chapin, 1947) that foetal immunization produces at least two types of antibodies in the maternal serum; one reacts with red cell antigens whilst the other, which cannot be detected by available techniques, reacts with antigens in tissue cells. A more simple hypothesis is that the same antibody reacts with both red cell and tissue antigens. On the other hand, Hsia, Allen, Gellis and Diamond (1952) believe that hyperbilirubinaemia following red cell destruction is responsible for injury to cerebral tissue, whilst Mollison (1951) claims that red cell haemolysis *per se* is sufficient to account for all the manifestations of the disease.

The presence in tissue cells of alcohol soluble antigens of the ABO system has been demonstrated by a number of workers (Witebsky and Okabe, 1927). Boorman and Dodd (1943) claimed to have shown the existence of antigens for the MN and Rh systems within tissue cells by absorbing antisera with washed tissue slices. This work has been criticized (Mollison, 1951) on the ground that the authors did not demonstrate conclusively the absence of red cell stromata from the tissues used for absorption. Van Bolhuis (1950) has also reported the presence of Rh antigens in placental tissue cells.

If tissue antigens are important in causing death in haemolytic disease of the newborn, current therapeutic methods, directed solely towards the haemolytic phase of the disease, are inadequate. This paper describes experiments which have been undertaken to confirm the existence of tissue antigens and to assess the rôle which they may play in the production of haemolytic disease of the newborn.

Materials.

Tissues. Autopsy specimens were obtained as soon as possible after death and were kept frozen at a temperature of —12° C. The majority of the specimens were obtained from infants who had died during or shortly after birth from haemolytic disease of the newborn, as confirmed by the finding of a positive direct Coombs test on the infants' red cells and by the presence of immune Rh agglutinins in the maternal serum. A few infants had died from other causes and their tissues, together with some specimens from both Rh positive and Rh negative adults, were used in control experiments.

Buffer solutions. Both Sörenson's citrate —HCl buffer (pH 3·3) and the M/15 phosphate buffer (pH 5·6) solutions were prepared according to Clark (1920).

Rh testing serum. "Incomplete" anti D serum was used in all experiments because it is more readily available than the "complete" anti-serum.

Anti-human globulin serum. Serum for the indirect Coombs test was obtained from the Commonwealth Serum Laboratories, Melbourne, and was prepared by the method described by Simmons (1946).

Methods.

It was realized that the removal of red cells and red cell stromata contained in tissue was most important. Homogenates of liver tissue prepared in the Waring blendor were found on phase-contrast microscopy to consist of small clumps of unbroken cells, single cells, isolated nuclei and cytoplasmic granules—a degree of dispersion which should permit removal of red cell stromata if a method was available. The method adopted in this work for the removal of stromata is based on the known fact that stromata are not readily centrifuged out of suspension in distilled water. Evidence will be presented to demonstrate the effectiveness of the procedure described below in removing red cell stromata.

Preparation of tissue. All tissue used in absorption and elution experiments was cut into small pieces approximately 0·5 to 1·0 cm. square and homogenized in the Waring blendor at full speed for 3 minutes with 50 ml. distilled water. The contents of the blendor were transferred to centrifuge tubes and, with the object of removing the stromata, the homogenate was washed 6 times with approximately 10 volumes of distilled water on each occasion. After each washing the homogenate was centrifuged at 1,500 *g*.

Detection of tissue antigens. A volume of anti-Rh serum was mixed with a small quantity of packed tissue homogenate prepared as described above. The mixture was placed in an incubator at 37° C. for 2 hours, and the supernatant serum was removed after centrifugation. This supernatant serum was serially titrated by the indirect Coombs test (Coombs, Mourant and Race, 1945) against red cells containing the corresponding antigen, using physiological saline for dilution. A titration of unabsorbed serum was always performed in parallel. After incubation at 37° C. for 60 minutes, the red cells were washed 3 times. A drop of appropriately diluted (usually 1 in 7) anti-human globulin serum was then added to each drop of washed cells on a white tile. The mixtures were examined for macroscopic agglutination at the end of 10 minutes.

Elution of antibody from tissues. Kidd (1949) described a method for elution of antibody from red cells by lowering the pH. This method was modified for use with tissues and formed the basis of the early work. Later a simpler method was devised which gave consistent results. The heat elution technique of Landsteiner and Miller (1925) was also employed.

Method 1. Tissue of infants who had died from haemolytic disease was prepared as described above. Citrate HCl buffer was added to the packed homogenate in an amount equal to one-quarter to one-half of its volume. The pH was adjusted to 3·3 and the mixture placed at 37° C. for 2 hours. The homogenate was then removed by centrifuging and the

pH of the supernatant eluate raised to 7·4 with NaOH solution. The eluate was tested as described below. All measurements of pH were performed with a pH electrometer.

Method 2. Homogenized and washed tissue of infants, prepared as described, was suspended in physiological saline solution instead of citrate HCl buffer. The mixture was incubated at 37° C. for 2 hours, after which time the supernatant fluid was removed and tested for the presence of antibodies. Throughout this procedure the pH was not altered and was approximately 7·0.

Method 3. Homogenized tissue suspended in an equal volume of physiological saline solution was heated at 56° C. for 10 minutes. Without delay the tubes containing the tissue were transferred to centrifuge buckets previously heated to 56° C., and after centrifuging the supernatant was removed immediately.

Testing of eluates. All eluates were tested for the presence of antibodies by the indirect Coombs test. It was found that most eluates haemolysed red blood cells used for testing but that this was prevented by the addition of group AB serum to the eluate. This phenomenon was not investigated, but it is probably due to extraction from washed tissue of "erythrocytase" (Maegraith, Findlay and Martin, 1943) or of lysolecithin (Ponder, 1948). The indirect Coombs test was performed by measuring twenty drops of the eluate into a 3-in. × 3/8-in. test tube and adding 5 drops of group AB serum and 2 drops of a suspension of group O Rh positive red cells. The tube was placed in an incubator at 37° C. for 60 minutes. The red cells were then washed three times with saline and resuspended in saline. One drop of the cell suspension was added to one drop of anti-human globulin (Coombs) serum on a slide and the mixture examined for the presence of agglutination. Controls of the reagents used in the test were always performed as follows:

(1) A direct Coombs test was performed on the cells used for testing the eluate by mixing a drop of the washed cells on a slide with a drop of anti-human globulin serum.

(2) The eluate with added group AB serum was tested by the indirect Coombs test against group O Rh negative cells.

(3) A mixture of 20 drops of physiological saline and 5 drops of group AB serum was similarly tested against group O Rh positive cells.

(4) The D negative test cells were mixed with incomplete anti D serum and tested with anti-human globulin serum after incubation and washing.

(5) The D positive cells were tested in a similar manner to the D negative cells.

No tissue was considered to contain antibodies unless the test with the eluate was positive and unless the first four controls were negative and the fifth positive.

Evidence that red cell stromata are removed from homogenates prepared as above. Previous attempts to demonstrate the presence of Rh antigens in tissue cells have been criticized (Mollison, 1951) on the ground that red cells may not have been removed from the tissue. The following experiments were performed to demonstrate that the methods used in this work to remove red cell stromata were adequate.

1. The red cells from 20 ml. of group O Rh positive blood were coated with incomplete D antibodies and the cells were found to give a strong positive Coombs test. The washed coated cells were then homogenized with 10 gm. of liver from an Rh negative subject and 50 ml. of distilled water. After being frozen at —12° C. for at least 12 hours, the mixture was washed 6 times with distilled water. Attempts were made to elute antibody into saline solution. The eluate gave negative indirect Coombs tests against D positive red cells. These results were confirmed on many occasions with different tissues, with varying amounts of red cells, employing the different techniques described earlier for elution of antibody and by using both frozen and unfrozen mixtures of tissues and red cells.

2. The homogenate prepared from 10 gm. of liver in 50 ml. of distilled water was washed 20 times with physiological saline solution. 10 ml. of oxalated blood and 40 ml.

of distilled water were then added to the homogenate and the mixture washed a further 6 times with distilled water. These latter washings were pooled, the pH lowered to 5·6 and the precipitate of red cell stromata weighed. A quantitative recovery of stromata was obtained when compared with the weight of the stromata precipitated from a further 10 ml. of oxalated blood and 40 ml. of distilled water. It was found that thorough preliminary washing of the homogenate with saline solution was necessary in this experiment because distilled water washing alone removed cytoplasmic granules and brownish-yellow crystals (possibly bilirubin) which were precipitated at pH 5·6. The granules and crystals were examined and identified with the phase contrast microscope.

RESULTS.

The presence of Rh antigens in tissue. It was demonstrated on many occasions that anti D is specifically absorbed from serum by homogenized, distilled-water washed tissues obtained from an Rh positive subject. A typical experiment was as follows:

Various tissues were obtained at autopsy from a D positive adult subject. 5 gm. (wet weight) of each tissue were homogenized and washed as described earlier. 1·5 ml. of incomplete anti D serum was added to each homogenate and, after absorption, the agglutinin was titrated by the indirect Coombs test. The results are shown in Table 1, from which it can be seen that all tissues absorb appreciable amounts of antibody.

TABLE 1.

Titration of incomplete anti D serum before and after absorption by treated homogenized tissues.

Serum absorbed with	Titre									
	2	4	8	16	32	64	128	256	512	1024
Liver	n.t.	+	+	+	—	—	—	—	—	—
Kidney	n.t.	+	+	+	—	—	—	—	—	—
Spleen	n.t.	+	+	+	+	—	—	—	—	—
Heart	n.t.	+	+	—	—	—	—	—	—	—
Brain	n.t.	+	+	+	+	—	—	—	—	—
Unabsorbed	n.t.	+	+	+	+	+	+	+	+	—

n.t. = not tested.

Attempts to distinguish quantitative differences between the amount of antigen in various tissues were not consistent. No evidence of non-specific antibody absorption by tissue homogenates was obtained. For example, liver tissue from a subject who was group B, CDe/cde, absorbed anti B, anti C and anti D, but did not absorb anti A or anti E.

The action of water and saline on tissue antigens. The method of preparing tissue homogenates was devised after it had been demonstrated that repeated washings of the homogenate with distilled water did not affect the capacity

of tissue to absorb antibody. On the other hand, use of physiological saline solution for washing resulted in loss of tissue antigen. This is shown in Table 2. The techniques of absorption and titration were as described earlier.

TABLE 2.

Titration of incomplete anti D serum before and after absorption with liver homogenate treated by different procedures.

Treatment of liver homogenate	Titre 2	4	8	16	32	64	128
1. Washed 3 times with distilled water	+	+	±	—	—	—	—
2. Washed 9 times with distilled water	+	+	±	—	—	—	—
3. Washed 6 times with distilled water and 3 times with saline	+	+	+	±	—	—	—
4. Washed 6 times with saline and 3 times with distilled water	+	+	+	+	+	—	—
Unabsorbed serum	+	+	+	+	+	+	—

Agglutination reactions are graded as +, ± or —.

This experiment would seem to have special significance. The loss of antigen in the saline washings would explain the failure of some of the early attempts to elute antibody from the tissues of infants who had died of haemolytic disease, Presumably antibody attached to antigen was removed during the saline washing prior to elution.

Elution of antibody from tissue antigens. The spontaneous elution of antibody from tissue into saline at pH 7·0 was an unexpected finding. To investigate this phenomenon further, Rh positive red cells coated with anti D agglutinins were washed 6 times with 10 volumes of physiological saline. A volume of saline solution equivalent to one quarter of the volume of the red cells was added and the mixture incubated at 37° C. for 2 hours. The supernatant saline was found to contain large amounts of anti D agglutinins which must have been previously attached to the red cell receptors. No agglutinins were detected in the saline used for the final washing of the coated cells.

As elution into saline was the simplest method it was generally used in this work, but all three methods were frequently employed on the same tissue with consistent results. The heat elution technique was the least satisfactory.

The results of elution on 13 cases are shown in Table 3. Twelve of the maternal sera contained D antibodies and the eluates were tested only for the presence of this antibody. Case No. 13 was the result of maternal immunization against the antigen c.

It must be emphasized that the antibodies were powerful in the eluates in only 2 or 3 instances, and that only once (Case No. 5) did the reaction equal in strength that of the positive control. On the other hand, weak (±) reactions

were obtained in two instances (Cases No. 11 and 13) with D negative cells. Owing to lack of material it was not possible to re-test these two eluates, but it is probable that the reactions were non-specific in nature. In three instances antibody could not be eluted from the tissues. No explanation can be offered for this failure. In the remaining 10 cases antibody was recovered from most, but not all, of the tissues investigated. The eluates from the liver and spleen gave positive results in all cases, but those from the kidney and brain were not invariably positive.

TABLE 3.

Elution of antibody from tissues of infants with haemolytic disease.

Case No.	Antibody in maternal serum	Reaction of eluate from tissue with test cells					Test Cells
		Liver	Kidney	Spleen	Heart	Brain	
1.	Anti D	+	+	±	+	+	O Rh + ve
		—	—	—	—	—	O Rh — ve
2.	Anti D and Anti C	+	+	+		±	O Rh + ve
		—	—	—		—	O Rh — ve
3.	Anti D and Anti C	—	—	—		—	O Rh + ve
		—	—	—		—	O Rh — ve
4.	Anti D	+		+		±	O Rh + ve
		—		—		—	O Rh — ve
5.	Anti D	+	+	+	+	+	O Rh + ve
		—	—	—	—	—	O Rh — ve
6.	Anti D and Anti C	+	+	±			O Rh + ve
		—	—	—			O Rh — ve
7.	Anti D	—	—	—	—	—	O Rh + ve
		—	—	—	—	—	O Rh — ve
8.	Twins						
	i. Anti D	+	+		—		O Rh + ve
		—	—		—		O Rh — ve
	ii. Anti D	+	—				O Rh + ve
		—	—				O Rh — ve
9.	Anti D	—					O Rh + ve
		—					O Rh — ve
10.	Anti D	±	±	+	±	±	O Rh + ve
		—	—	—	—	—	O Rh — ve
11.	Anti D	±	+	+		±	O Rh + ve
		—	±	—		—	O Rh — ve
12.	Anti D	+	—	±	±	—	O Rh + ve
		—	—	—	—	—	O Rh — ve
13.	Anti c	+	—	+	+	—	O cde/cde
		—	—	±	—	—	O CDe/CDe

DISCUSSION.

The experiments reported in this paper show:

1. That tissue cells contain Rh antigens, thus confirming the work of Boorman and Dodd (1943). It has long been known that ABO antigens are present in the tissue cells, so the finding of the Rh antigen was not unexpected.

2. That the Rh antibody derived from the mother may become attached to the Rh antigen in the tissue cells of infants suffering from haemolytic disease of the newborn.

The true significance of this second finding must await further evidence, but the hypothesis is advanced that some of the lesions of haemolytic disease, notably those of the liver and brain, are due to the union of the Rh antibody with its corresponding antigen in the tissues of these organs. Admittedly, there is no direct evidence that this union is injurious to the cell, but there is some indirect evidence of a harmful effect. For example, Medawar (1946) has shown that grafted epidermis fails to proliferate in animals who have previously received grafts from the same donor animal. Medawar suggests that this is due to the action of antibodies formed against epidermal antigens. It is true, however, that the situation in haemolytic disease is somewhat different, because in Medawar's experiments it is the antigens of the graft which are foreign, whereas in haemolytic disease it is the antibodies which are foreign.

Some of the presenting signs of haemolytic disease, such as the anaemia and the jaundice, are clearly due to red cell destruction consequent on the union of the antibody with the antigen of the red cell. But the lesions in the liver and brain are more difficult to explain. That they are due to tissue anoxia consequent on red cell destruction is not entirely convincing. The findings reported in this paper suggest another possibility—that they are due to the injurious effect of the same union of antibody and antigen, but in this case on the tissue cells of the organ and not on the red cells.

From the practical point of view tentative acceptance of the hypothesis concerning the action of the Rh antibody on the tissue cells necessitates a review of the therapy of haemolytic disease of the newborn. Ideally, this therapy should be directed towards preventing any injurious effects of such a union to either red blood cells or tissue cells. Transfusion of blood is, of course, necessary to prevent tissue anoxia, but *per se* does not remove antibody attached to tissue cells. On the other hand, replacement transfusion, although a laborious method of providing viable red cells, removes unattached antibody in the infant's serum and may possibly cause elution of some antibody attached to tissue cells. This elution, however, is not complete because infants dying after replacement transfusion have still had antibody adherent to tissue cells (Cases 1, 4 and 11). The question must therefore be raised as to whether a more active elution of

tissue antibody would not be achieved *in vivo* if Rh positive blood were transfused. At the same time it must be admitted that there are theoretical and practical objections to the use of Rh positive blood.

SUMMARY.

A technique has been devised for removing red cells from tissue, and using this technique it has been shown that the Rh antigen is present in the tissue cells themselves.

After removing the red cells from the tissue, Rh antibodies have been eluted from the organs of infants who have died from haemolytic disease.

The pathogenesis of haemolytic disease of the newborn is discussed in the light of these findings.

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LYSOGENICITY AND LYSIS PATTERNS IN THE SALMONELLAS

2. FURTHER TESTS WITH *S. ADELAIDE*[1]

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Atkinson *et al.* (1952) described the lysis and lysogenicity patterns of 48 Australian strains of *S. adelaide*. By the method of cross-plating these strains among themselves, twelve proved to be lysogenic and provided phages which Atkinson and Geytenbeek (1953) subsequently divided into four types, 1a, 1b, 2a and 2b. On the basis of lysis pattern and phage carriage, 47 of the 48 strains were divided into four groups, 1a, 1b, 2 and 3. Shortly after this work had been reported, 24 new strains of *S. adelaide* were obtained from Dr. E. Schmid (1951) of Colombo, Ceylon, who had found numerous strains of *S. adelaide* among Ceylon Salmonellas typed in his laboratory. We are most grateful to Dr. Schmid for sending these cultures. The Ceylon strains revealed a new phage which was present in all of the 48 Australian strains and also in nine additional strains. Seven new strains of *S. adelaide* were obtained from Dr. P. R. Edwards of Atlanta, U.S.A., to whom we are most grateful. The new phage was detected in six of these strains, five of which also yielded a second new phage. The present paper describes the lysis and lysogenicity patterns now discernible in our group of 89 strains of *S. adelaide*.

METHODS.

Strains were tested for lysogenicity by the cross-plating method as described previously (Atkinson *et al.*, 1952). Tests for range of action of phages were made by patch-testing using either an unheated centrifuged extract of an agar culture of a suitable mixture of the lysogenic strain and one of the indicator strains 14 or Cey4, or a single plaque preparation propagated on one of the indicator strains.

EXPERIMENTAL.

Lysogenicity tests.

The 24 new Ceylon strains, which were labelled Cey 1–24, were cross-plated with the indicator strain 14 to detect *S adelaide* phages of types 1a, 1b, 2a or 2b. No phages were detected in the Ceylon strains but all were lysed by strain 14 in which no phage had previously been detected. The new phage, designated

[1]This work was assisted by a grant from the National Health and Medical Research Council, Canberra.

Ad14, was isolated and propagated on strain Cey4. The 48 Australian strains previously described (Atkinson *et al.*, 1952) as consisting of 36 non-lysogenic and 12 lysogenic strains were tested with phage Ad14 and all were resistant. They were then tested for lysogenicity by cross-plating on strain Cey4. The 36 previously non-lysogenic strains (1,3,4,5,6,7,8,9,10,12,14,17,18,19,21,23,25,26, 27,28,30,31,32,33,34,35,36,38,41,42,46,48,50,51,52,53) all carried a phage detectable by the indicator strain Cey4 and producing plaques similar to phage Ad14. Single plaque preparations from representative strains 1,6,7,8 and 32 failed to lyse the indicator strain 14 or strains 1,6,7,8 or 32. These phages were probably identical with phage Ad14. The twelve lysogenic strains (2,13, 15,16,20,37,39,40,43,45,47,49) when cross-plated with strain Cey4, all yielded phage preparations producing two types of plaque, one of which was similar to phage Ad14, the other resembled the phage already recorded for each strain when tested on the indicator strain 14. From all these phage preparations single plaques of each type were propagated on strain Cey4 and tested on the two indicator strains 14 and Cey4 and the strain from which the phage came. From each preparation the isolated plaques resembling phage Ad14 produced lysis of strain Cey4 but not of strain 14 or the parent strain, and thus were like phage Ad14. The second type of plaque lysed both strain 14 and strain Cey4 and thus resembled the phage already found in each strain. The possibility remained, however, that the second phage revealed by strain Cey4 was not identical with that already isolated on strain 14. To investigate this possibility, the lysis patterns of single plaque preparations of the two phages detected by strain Cey4 were determined for representative Australian strains and compared with the lysis patterns of the original phages isolated on strain 14. To distinguish phage preparations isolated on strain 14 from those isolated on strain Cey4, the conventional method of numbering with the lysogenic strain number followed by the indicator strain number has been used in Table 1. The two plaque types found on Cey4 are indicated by the letters A and B, A for the plaques resembling Ad14 and B for the plaques resembling the phage already detected by strain 14. Results are given in Table 1.

The results in Table 1 indicated that, of the two phage preparations A and B from each lysogenic strain on Cey4, preparation A resembled Ad14 in lytic pattern, and preparation B resembled the phage already detected in that strain by the indicator strain 14 and was not another new phage. The reactions of phage Ad14 with a wide range of strains of *S. adelaide* are given in Table 2. All except the Ceylon strains were resistant to this phage.

Thus the 48 Australian strains were all lysogenic and carried phages which were detectable by the indicator strain Cey4 and resembled Ad14. In 12 of these strains a second phage was also present. It was detectable by both the indicator strains 14 and Cey4 and appeared to be identical with the phage already found in each of these strains.

TABLE 1.

Comparison of range of action of phages isolated on indicator strains 14 and Cey4 from S. adelaide *strains carrying more than one phage.*

Phage tested	Strains used in test					
	2	14	20	37	S273	Cey4
2/14(Ad2)	—	+	+	+	+	+
2/Cey4A	—	—	—	—	—	+
2/Cey4B	—	pls	pls	pls	pls	pls
13/14	+	+	+	—	—	+
13/Cey4A	—	—	—	—	—	+
13/Cey4B	+	+	+	—	—	+
15/14	+	+	+	—	—	+
15/Cey4A	—	—	—	—	—	+
15/Cey4B	+	+	+	—	—	+
37/14(Ad37H)	+	+	+	—	—	+
37/Cey4A	—	—	—	—	—	+
37/Cey4B	+	+	+	—	—	+
47/14	+	+	+	—	—	+
47/Cey4A	—	—	—	—	—	+
47/Cey4B	pls	pls	pls	—	—	+
20/14(Ad20)	+	+	—	+	+	+
20/Cey4A	—	—	—	—	—	+
20/Cey4B	pls	+	—	pls	pls	pls
S273/14(Ad273)	+	+	+	+	—	+
S273/Cey4A	—	—	—	—	—	+
S273/Cey4B	+	+	+	+	—	+
S274/14	+	+	+	+	—	+
S274/Cey4A	—	—	—	—	—	+
S274/Cey4B	+	+	+	+	—	+
Ad14	—	—	—	—	—	+

Phage preparations used undiluted. + = confluent or semi-confluent lysis; — = no lysis; pls = plaques.

Ten new Australian strains (54,55,56,57,58,59,67,92,93,94) were examined for lysogenicity on strains 14 and Cey4. None was lysogenic on strain 14 and all except strain 67 were lysogenic on strain Cey4. The plaques produced by the phages isolated from the lysogenic strains 54,55,56,57,58,59,92,93,94 resembled Ad14 plaques. These phages appeared to be identical with phage Ad14. These 9 lysogenic strains thus resembled the 36 strains described above, which were shown to carry phage Ad14. Strain 67 was non-lysogenic by our present tests, but failed to lyse with phage Ad14. It was therefore unlike any other strain so far found.

Strain Cey4 failed to reveal any phage itself during the cross-plating tests with the 48 Australian strains. In a further search for phage in the 24 Ceylon strains, they were cross-plated among themselves in every combination, but none was lysogenic. Thus the 24 Ceylon strains formed a group which, by all available tests, was not lysogenic and was susceptible to all phages so far found (see Table 2).

Six new American strains (our numbers S273, S274, S275, S276, S277, S278) and one new English strain (our number S279) were investigated for lysogenicity by cross-plating on strains 14 and Cey4. On strain 14, five strains (S273, S274, S275, S276 and S277) were lysogenic and two (S278 and S279) had no effect. Phage preparations Ad273, Ad274, Ad275, Ad276 and Ad277 from the five lysogenic strains gave similar plaques and failed to lyse these five strains, but lysed the Australian strains 2, 20 and 37 carrying phages of types 1a, 1b and 2a respectively, acting on strain 14 (see Table 2). These results suggested that the five phages were similar and represented a type of *S. adelaide* phage not so far encountered. On strain Cey4, six strains (S273, S274, S275, S276, S277, S279) were lysogenic and one strain (S278) had no effect. The lysogenic group included the five strains already shown to be lysogenic on strain 14. They produced two types of plaque on strain Cey4, one resembling phage Ad14, the other resembling phage Ad273. From all these strains single plaque preparations of each plaque type were made on strain Cey4 and tested on the parent strain and strains 14 and Cey4. From each lysogenic strain were thus obtained two phages, one probably identical with Ad14 and lysing only strain Cey4, the other resembling phage Ad273 and lysing strains 14 and Cey4 but not strain S273 (see Table 1). From strain S279 only one plaque type resembling Ad14 appeared on strain Cey4. This phage, Ad279, like phage Ad14, lysed only strain Cey4 (see Table 2). The lysis patterns of phages Ad273 and Ad279, representing the two types of phage found here, are given in Table 2. All except the five American strains and S278 lysed with phage Ad273. Only the Ceylon strains lysed with phage Ad279, which was therefore identical with phage Ad14.

Thus five of the American strains and the one English strain were lysogenic and resembled the majority of Australian strains in carrying a phage which appeared identical with phage Ad14 and was detectable by the indicator strain Cey4, but not by the indicator strain 14. In the five American strains a second phage was also present and was detectable by both the indicator strains 14 and Cey4. This phage, which appeared to be the same for the five strains, differed in lysis pattern from any phage so far found in Australian strains. One American strain (S278) yielded no phage on either of the indicator strains 14 or Cey4 and was very rough.

Lysis tests.

All the Ceylon and American strains, the English strain and the 10 new Australian strains were tested with *S. adelaide* phage types 1a, 1b, 2a and 2b. They were also tested with phage Ad14 representing the new type of *S. adelaide* phage found in all but one of the Australian strains, the English strain, and five out of the six American strains, and phage Ad273 representing the second new type of *S. adelaide* phage found in five of the American strains. The results of

these tests are given in Table 2, which also includes, for comparison, the Australian strains 2 (group 1a), 20 (group 1b) and 37 (group 2) which carry phage types 1a, 1b and 2a respectively, and strains, 3,6,7,8 and 14 (group 3) and 32 previously thought to be non-lysogenic, but now shown to carry phage like Ad14.

TABLE 2.

Reactions of Ceylon, American, English and Australian strains of S. adelaide *with representatives of all known* S. adelaide *phages.*

Strain tested	Phages used in test						
	Type 1a (Ad2)	Type 1b (Ad20)	Type 2a (Ad37H)	Type 2b (Ad37C)	Ad14	Ad273	Ad279
Ceylon strains							
Cey 1–24 (24 strains)	+	+	+	+	+	+	+
American strains							
S273	+	+	—	—	—	—	—
S274	+	+	—	—	—	—	—
S275	+	+	—	—	—	—	—
S276	+	+	—	—	—	—	—
S277	+	+	—	—	—	—	—
S278	—	—	—	—	—	—	—
English strain							
S279	+	+	+	+	—	+	—
Australian strains							
54	+	+	+	+	—	+	—
55	+	+	+	+	—	+	—
56	+	+	+	+	—	+	—
57	+	+	+	+	—	+	—
58	+	+	+	+	—	+	—
59	+	+	+	+	—	+	—
67	+	+	+	+	—	+	—
92	+	+	+	+	—	+	—
93	+	+	+	+	—	+	—
94	+	+	+	+	—	+	—
** 2 (group 1a)	—	+	+	+	—	+	—
**20 (group 1b)	+	—	+	+	—	+	—
**37 (group 2)	+	+	—	—	—	+	—
** 3 (group 3)	+	+	+	+	—	+	—
** 6 (group 3)	+	+	+	+	—	+	—
** 7 (group 3)	+	+	+	+	—	+	—
** 8 (group 3)	+	+	+	+	—	+	—
**14 (group 3)	+	+	+	+	—	+	—
**32 (ungrouped)	—	pls	—	—	—	pls	—

Phage preparations used undiluted. + = confluent or semi-confluent lysis; — = no lysis; pls = plaques.

** These strains were previously recorded by Atkinson and Geytenbeek, 1953.

With the type phages 1a, 1b, 2a and 2b, lysis patterns were obtained by which all strains, except 32 and S278, could be allocated to one of the groups suggested by Atkinson and Geytenbeek (1953). Thus all the Ceylon strains (Cey 1-24) the English strain (S279) and the 10 new Australian strains (54-59, 67,

92–94) showed the lysis pattern of group 3, and five American strains (S273–S277) showed the lysis pattern of group 2. However, further differentiation of group 2 and group 3 into two parts was obtained when the lysis patterns with the two new phages Ad273 and Ad14 were included. Thus the group 2 strains, which were all resistant to phage Ad14, could be divided into the Australian strain 37, which was lysed by phage Ad273, and the American strains which were resistant. The other Australian strains (13,15,16,39,40,43,45,47 and 49) of group 2, previously described but not included in Table 2, were tested and also lysed with phage Ad273. Thus the American and Australian strains could be differentiated within group 2. The group 3 strains were further divided on the action of phage Ad14. The Ceylon group were all lysed by phage Ad14, but the other strains of group 3 were resistant.

With eight exceptions (strains S273, S274, S275, S276, S277, S278, 32 and 67) the lysis pattern was directly related to phage carriage. Thus the lysogenic strains were resistant to the phage or phages they carried and susceptible to the other phages, and the non-lysogenic strains were sensitive to all phages. Of the exceptions, lysogenicity was not detected in strains 67 and S278, and their failure to lyse must be attributed to other causes; strain 32 was lysogenic, carrying phage like Ad14, but was resistant not only to this phage but to most of the other phages also. This resistance was not due to carriage of phage Ad14 as all other strains carrying this phage lysed with some or all of the other phages. Strains S273–S277 were lysogenic, carrying two phages, one like Ad14 and the other like Ad273. The lysis pattern of these strains was unusual in that they were resistant not only to phages Ad14 and Ad273, but also to the type 2a and 2b phages. These results suggested a close relationship between the type 2 phages and phage Ad273. To confirm this result other type 2 phages, Ad13H, Ad15H, Ad47H, Ad13C, Ad15C, and Ad47C, were tested on strain S273 which was resistant. However, strain 37 and the other group 2 strains (13,15,16,39,40,43,45,47 and 49) carrying phage type 2a were susceptible to phage Ad273.

DISCUSSION.

In Paper I of this series only 12 out of 48 Australian strains of *S. adelaide* were found to be lysogenic. The present paper records the finding of a new phage responsible for lysogenicity in all these 48 strains and in 15 new strains. In the earlier work lysogenicity was sought using all available *S. adelaide* strains as indicators (no other Salmonella types were included), and as they all carried the new phage, they were resistant to its action and so failed to reveal it. However, the 24 new Ceylon strains of *S. adelaide* did not carry this phage and thus provided a group of sensitive indicator strains which readily detected it in the Australian strains. One English and five American strains also carried

this phage. The Ceylon strains have so far proved non-lysogenic, but may possibly carry a phage also present in all other *S. adelaide* strains so far tested and therefore not detectable. Further tests with other Salmonella types as indicators will be carried out and may detect phages not detectable by any *S. adelaide* strain. Thus phages carried by numerous strains are most likely to be missed, and we have the paradox that the commonest phages of lysogenic bacteria are those most difficult to locate. The five lysogenic American strains also carried another new phage which distinguished them from all other strains of *S. adelaide*. They were all obtained from one laboratory in 1949. Including the 12 lysogenic strains previously described, we thus have 17 strains carrying at least two types of phage, one of which was the same for all strains, and the other characteristic of the strain carrying it. We also have 46 strains all carrying one similar phage. Thus on lysis and lysogenicity patterns, *S. adelaide* strains may be divided into six groups consisting of groups 1a, 1b, 2 and 3, already suggested by Atkinson and Geytenbeek (1953), and two further groups represented by the non-lysogenic Ceylon strains and the American strains carrying phage of the Ad273 type. Only three strains (S278, 67 and 32), out of the 89 investigated, failed to fit into any of these groups. This grouping scheme will be presented more fully, together with the characteristics of the new phages, in a separate paper.

SUMMARY.

A group of 89 strains of *S. adelaide*, consisting of 58 Australian strains, 24 Ceylon strains, six American strains and one English strain, was examined for lysogenicity. Two new phages were found. By use of the indicator strain Cey4 all (except one) of the Australian strains, five of the American strains and the English strain were shown to be lysogenic and to carry a similar phage of a new type. No phage was detectable in the Ceylon strains.

Twelve of the Australian strains and five of the American strains also carried a second phage which differed for different strains and served to divide the strains into groups. The second phage of the American strains was a new type not previously recorded for *S. adelaide* and detectable by the indicator strain 14.

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A MORPHOLOGICAL STUDY WITH THE ELECTRON MICROSCOPE OF SECTIONS OF THE NORMAL MOUSE PANCREAS

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Publications are now appearing with increasing frequency on the appearances seen with the electron microscope in thin sections of biological tissues. The present paper reports such a study of the normal mouse pancreas.

METHODS.

Male albino mice aged 6–8 weeks were used. They were fed on standard compressed mouse nuts, with water *ad lib.*, and were not deprived of food or water prior to sacrifice. As soon as possible after death the pancreas was removed into a drop of 1 p.c. osmic acid fixative adjusted to pH 7·4 with 0·028 M acetate-veronal buffer (Palade, 1952). The organ was then divided in the drop of fixative on a card into approximately 1 mm. cubes, which were transferred into a larger volume of the fixative mixture for 4 hours at room temperature in the dark. They were then washed for 1 hour in each of 2 changes of distilled water, and passed through graded ethyl alcohols as follows: 25 p.c. alcohol 1 hour; 50 p.c. alcohol 1 hour; 75 p.c. alcohol overnight; 95 p.c. alcohol 1 hour; absolute alcohol ½ hour in each of 2 changes; equal parts of absolute alcohol and methacrylate 1 hour; pure methacrylate ½ hour in each of 2 changes. They were then embedded in gelatine capsules in a further change of methacrylate, and left to harden, without prepolymerization, in an oven at 47° C. All changes of methacrylate contained the catalyst (2 p.c. benzoyl peroxide) and the proportions of the methyl and butyl monomers used varied between 2:7 and 3:7 according to the batch.

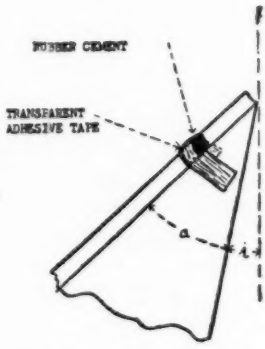
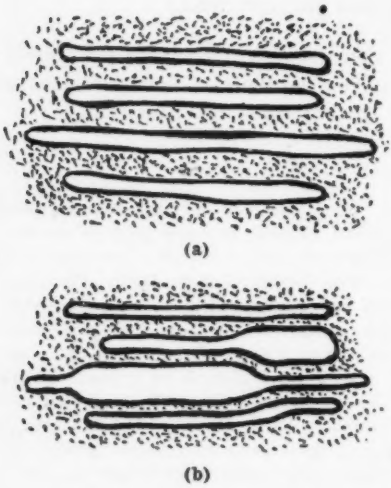


Fig. 1. The arrangement of the glass knife.

Sections were cut on to water with a geared-down rotary Leitz microtome mechanically driven at a rate of 45/minute. The glass knives first recommended by Latta and Hartmann (1950) were used. It was found most convenient not to attach a bath to the knife, but to hang a drop of water from the knife edge, holding it in place with a simple dam made from a small piece of transparent adhesive tape folded longitudinally into three with the sticky side outwards, and with its upper border, which was placed about 7 mm. from the knife edge, covered with rubber cement. This arrangement is shown diagrammatically in Fig. 1. The inclination of the knife (angle i in Fig. 1) was not found to be particularly critical, but the angle of the glass wedge itself (angle a in Fig. 1)

was important. If the glass was cut at an angle greater than the 45° recommended by Latta and Hartmann, most sections were spoiled by an intractable fine transverse folding which affected particularly the denser portions of the cells. An angle of 35–40° gave the best results.

When sufficient sections had been collected on the drop of water the microtome was stopped, and the water carefully removed with a fine pipette and replaced by 50 p.c. acetone which, as it ran under the raft of sections, materially assisted their flattening. The acetone was removed in its turn and replaced again by water, from which the sections were picked up on a copper grid coated with a collodion film. In order to enable the grid to enter the surface film of the relatively small volume of water on the knife, it was necessary first to place a small drop of water on the upper surface of the film-bearing grid as it was held in the forceps before touching the grid to the surface of the water on the knife. After picking up the sections, the grids were set aside to dry on lens paper under cover. The embedding medium was not removed before inserting the grids into the microscope (Philips) for examination.



(a)

(b)

Fig. 2. The formation of vacuoles from cytoplasmic double membranes.

RESULTS.

Acinar cells[1].

These cells have large, regular, round or ovoid nuclei bounded by a double nuclear membrane. The two components of this membrane lie approximately 30–40 mμ apart, and are usually parallel (Fig. 4). The outer component is continuous but can sometimes be seen, apparently as the result of slight damage to the cell, to be slightly elevated over a little distance (Fig. 4). Much more rarely the reverse occurs—a depression of the inner component. The outer component appears somewhat less dense and less resistant to degenerative changes than the inner, as in some slightly damaged cells it is in places incomplete, breaking up into rows of granules. Occasionally a cytoplasmic membrane appears to arise from the outer component of the nuclear membrane. This may be artefact.

The nucleoplasm is finely granular, the granules varying somewhat in size. Nucleoli are prominent, often multiple, and vary greatly in size and density even in the same nucleus. The smallest nucleoli, which are usually seen applied

[1]*Note added in proof.* While this paper was in the press, a comprehensive report by Weiss (J. exp. Med., 1953, 98, p. 607) has appeared concerning the fine structure of the cytoplasmic double membranes of pancreatic acinar cells. These are called by Weiss "ergastoplasmic sacs," and he presented evidence on their formation and appearance in various stages of cell activity, and their relation to the formation of secretory granules. The present findings do not appear to conflict with those of Weiss.

to the under surface of the nuclear membrane (Fig. 4) appear as little more than slightly darker areas in the nucleoplasm, though with a tendency to an irregular thread-like internal structure on close inspection. Larger nucleoli—and in the pancreatic acinar cells they are often very large—are dense structures with a "woolly" non-homogeneous appearance (Fig. 5). No nucleolar membranes can be seen.

Fig. 3. Acinar cells, low power view.

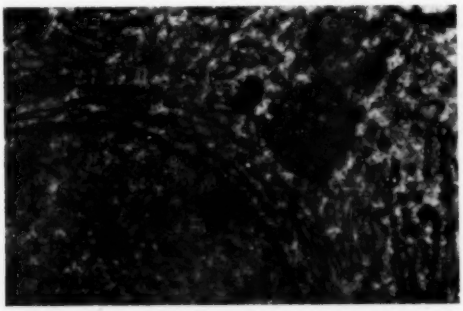


Fig. 4. Portion of an acinar cell, showing the nucleus at the lower left with a mitochondrion above it. Many cytoplasmic membranes are present.

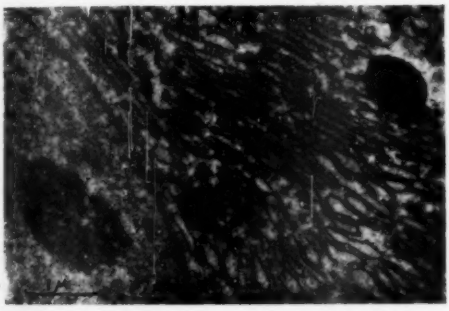
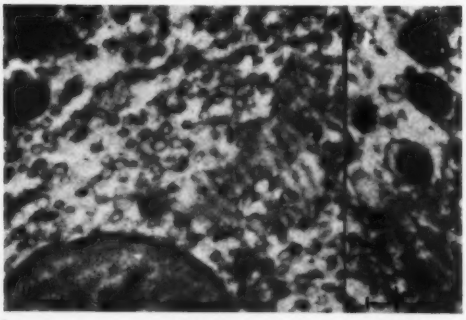


Fig. 5. Portion of an acinar cell. A large nucleolus at the lower left, a zymogen granule at the upper right. Many cytoplasmic double membranes are present.

Fig. 6. Portion of an acinar cell. Vacuolation of cytoplasmic double membranes is occurring.

The cytoplasm of the acinar cell presents a complex appearance. Those portions which in stained sections appear basophilic with the light microscope, contain a very large number of thin double cytoplasmic membranes with closed ends (Figs. 4–6). These membranes have already been noted in the pancreatic acinar cell by Dalton (1951), who thought they might perhaps be artefacts of fixation or embedding. However, Sjöstrand (1953) has seen them in frozen-

dried material, which indicates that they probably have a real existence. In cells apparently resting the two components of the cytoplasmic membranes lie rather uniformly parallel to one another, and some 30–60 mμ apart. The spacing is, however, very variable, and in many cases the components of the membranes can be seen to separate, usually to form a small vacuolar structure (Fig. 6). Sometimes, however, the cavity, like the space between the unseparated membranes, contains material of approximately the same density as the cytoplasm, though of more homogeneous texture (Fig. 4). The separation often appears to begin in a localized region of the double membrane, either terminally or more or less centrally, or both. When separation occurs in adjacent double membranes the outer layers of each come together and lie parallel to each other, with a thin layer of intervening cytoplasm, producing another type of double-lined appearance (Figs. 2 and 5).



(a) (b)

Fig. 7. Small oval cytoplasmic vacuoles are shown in (a). In (b) there is what appears to be a partially emptied zymogen granule.

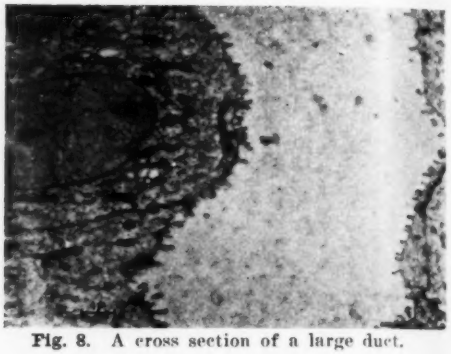


Fig. 8. A cross section of a large duct.

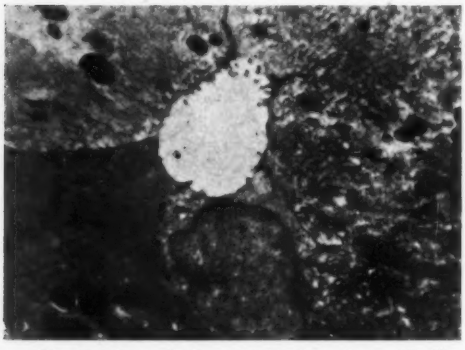


Fig. 9. A small duct at its junction with the acinar cells.

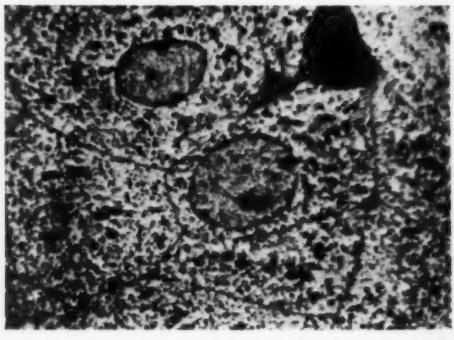


Fig. 10. Islet of Langerhans.

In some preparations the components of the cytoplasmic membranes can be resolved into rows of irregular spaced dots, but this appearance is probably artefactual, representing a minor degree of damage to the structure.

The less basophilic (usually upper) portions of the cytoplasm of the acinar cell contain fewer double membranes, and in some cases many small oval vacuoles (Fig. 7a). This is not invariable however. Fig. 9 shows an acinar cell with a dense aggregation of cytoplasmic membranes immediately beneath the free border of the cell.

Mitochondria are numerous and prominent in acinar cells. They are rounded, ovoid or rod-shaped, and show transverse or irregular internal membranes. These appearances in mitochondria have been already described in detail by Sjöstrand (1953). Zymogen granules are conspicuous. They have a thin outer membrane which could not be resolved into two components, and a dense interior in which no structural details could be made out as the contents of the granules seemed peculiarly susceptible to artefactual splitting. In a few cases what appeared to be a partially empty zymogen granule was seen (Fig. 7b).

Ducts.

Many ducts of all sizes were seen. The epithelium is irregularly flattened in the larger ducts, and composed of more cuboidal and barrel-shaped cells in the smaller ducts, the epithelium resting on a small amount of collagenous tissue. In all ducts, but more conspicuously in smaller ducts, the epithelial cells show scattered thin finger-like projections from their free borders into the lumen of the duct (Figs. 8 and 9). These projections are not cilia, being clearly seen at higher magnification to be prolongations of the peripheral cytoplasm. Furthermore, as in many cases they can be seen cut across, it is highly probable that they are not ridges (Fig. 9). It is, however, possible that they are artefacts produced by shrinkage during fixation or processing. Similar projections can also be seen in Fig. 9 on the free (ductal) border of acinar cells.

Cytoplasmic double membranes are scanty in duct cells, and mitochondria are not numerous. The nuclei of duct cells, though usually rounded or ovoid, are less regular in form than those of the acinar cells, often showing a slightly sinuous outline and not rarely quite deep indentations (Figs. 8 and 9). Nucleoli are small and not usually dense.

A few examples of the junction of ducts and acini were seen. One of these is illustrated in Fig. 9, which shows in the lower part a duct-type cell (centro-acinar cell) with an irregular nucleus and a rather clear cytoplasm wedged between two much darker acinar cells containing zymogen granules.

Islets of Langerhans.

The islet cells are polyhedra with very thin walls (Fig. 10). Their round or ovoid regular nuclei may contain more than one nucleolus, some quite dense. The cytoplasm is clear, with only an occasional cytoplasmic double membrane,

but there are numerous ovoid or filamentous mitochondria of typical structure. The islet-cell granules are small spherical dense bodies measuring approximately 65 mμ to 150 mμ in diameter.

SUMMARY.

Thin sections of the normal mouse pancreas have been examined in the electron microscope after fixation of the organ in buffered 1 p.c. osmic acid and embedding in a mixture of methyl and butyl methacrylates.

The appearances of the acinar cells, duct cells and islet cells are described and illustrated.

Acknowledgments. I am grateful to Dr. S. G. Tomlin, Reader in Biophysics, for the use of the electron microscope; to Mr. Ian McLean, B.Sc., for much assistance in its operation; and to Mr. R. Jones for technical assistance.

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OBSERVATIONS ON THE STRUCTURE OF THE RENAL GLOMERULUS OF THE MOUSE REVEALED BY THE ELECTRON MICROSCOPE

by R. T. W. REID

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(Accepted for publication 26th October, 1953.)

The development of the electron microscope and the ability to cut ultra-thin sections has enabled investigators to study the minute histology of many tissues. Some interest has been shown in the structure of the kidney, including the glomerulus.

The glomerular structure described by Pease and Baker (1950) was similar to that determined by the light microscope (McGregor, 1929; Bensley and Bensley, 1930). The glomerular tuft consisted of a basement membrane which separated capillary endothelial cells from the visceral layer of Bowman's capsular epithelium. In addition, Pease and Baker (1950) described a system of ridges and projections on the outer surface of the basement membrane from which they were considered to arise. In their opinion the capillary endothelial cells did not completely cover the inner aspect of the basement membrane, nor the visceral layer of Bowman's epithelial cells entirely cover it externally. They thought fixation artefacts might produce the small spaces between the epithelial cells and the membrane.

Dalton (1951) interpreted the electron microscope appearances somewhat differently. The endothelial cells of the capillary loops formed a complete internal lining to the basement membrane, and the complex processes external to it were considered to be cytoplasmic projections of the visceral epithelial cells.

Oberling, Gautier and Bernhard (1951) thought that projections from the basement membrane interdigitated with processes from the epithelial cells.

Rinehart, Farquhar, Haw Chan Jung and Abul-Haj (1953) agreed with Dalton (1951) that the complex "fronds" were processes of the epithelial cells and were not derived from the basement membrane. They thought, however, that delicate processes derived from the epithelial cells pierced the basement membrane at regular intervals and appeared on the endothelial aspect of the loops.

It seemed advisable to attempt to clarify the conception of normal glomerular structure by further electron microscope studies, especially if alterations produced by disease are to be understood.

The mouse was chosen in preference to the rat because of the comparative ease with which glomeruli could be detected in kidney sections and because of the frequent occurrence of pyelonephritis in the rat colony.

Materials and Methods.

Adult male white mice were killed by decapitation or a blow on the head. The kidneys were not perfused with the fixative, but were removed rapidly and thin slices of cortex reduced to cubes approximately 2 mm. square in a drop of 1 p.c. aqueous osmic acid fixative buffered at pH 7·4 by a sodium acetate-veronal solution (Palade, 1952). The tissue was immediately placed in fixative for 4 hours, dehydrated by passage through graded solutions of ethyl alcohol, embedded in a mixture of butyl and methyl methacrylate in the proportions of 7:4 and polymerized slowly at 47° C.

The tissue was cut with glass knives on a Leitz microtome fitted with reduction gears to produce sections 0·1 μ thick and the sections mounted on copper grids coated with a film of collodion.

Sections were viewed with a Philips electron microscope.

RESULTS.

Examination of the electron micrographs showed that the glomerular loops were composed of three components.

The main portion consisted of a homogeneous membrane approximately 0·1 μ thick when viewed in cross section. Apart from a delicate fibrillary character noted in some areas, it appeared to be the same density throughout with no pores or penetrations in its substance (Fig. 4).

Outside the membrane were the epithelial cells which were, to a greater or lesser extent, closely applied to it. The cytoplasm of these cells in places projected and impinged on the adjacent glomerular basement membrane either as a mass sending out finger-like processes to form a series of arcades or coming into intimate contact with the membrane for a variable distance (Fig. 3). Where contact occurred, the processes were of differing density to the basement membrane and ended abruptly on the surface with no penetration of its substance (Fig. 4). Processes apparently projecting from the basement membrane and appearing to have no connection with epithelial cells could be explained by the plane of section not coinciding with that of the cell of origin. Occasionally complex patterns were formed by the interdigitation of many processes.

As far as could be seen, the entire external surface of the basement membrane was covered by the cytoplasm of the visceral epithelial cells. Potential spaces between arcades and projections, and between complex interdigitations, were possibly accentuated during fixation and preparation of the tissue.

The nuclei of the epithelial cells were mainly oval in section and characteristically contained one round nucleolus, although two or more were occasionally present (Fig. 3). The nuclear membrane was distinct, and in some areas a less distinct membrane was visible parallel to it in the adjacent cytoplasm of the cell.

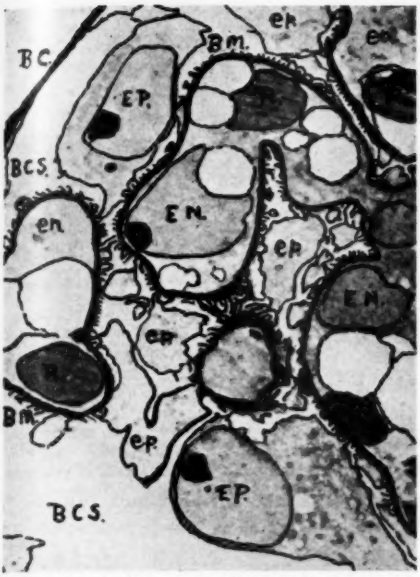

Fig. 1. A diagrammatic representation of Fig. 2 showing a segment of mouse glomerulus. The epithelial cells (EP) and cytoplasmic extensions (ep) are separated from the endothelial cells (EN) and their cytoplasm (en) by the basement membrane (BM) with processes on the outer surface. A few red cells (R) are inside the glomerular loops. Portions of Bowman's capsule (BC) and capsular space (BCS) appear in the left upper and lower quadrants.

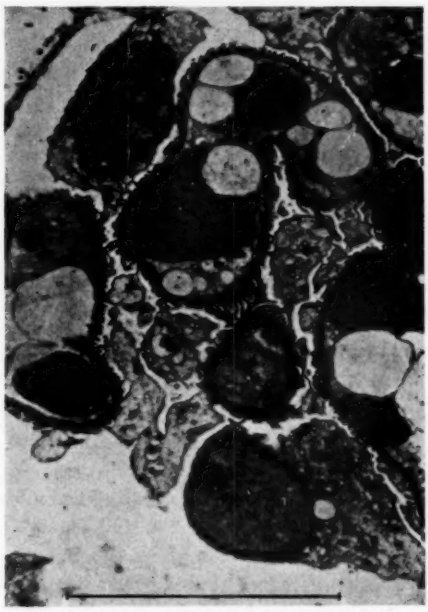

Fig. 2. An electron photomicrograph of a segment of mouse glomerulus. The distance between markers at the bottom of the illustration is 10 μ.

Fig. 3. An enlarged portion of Fig. 2 showing nucleus, nucleolus and cytoplasm of an epithelial cell. The cytoplasm is forming an arcade close to the basement membrane of a glomerular loop. Part of an endothelial nucleus and cytoplasm are seen in the lower left of the illustration. Two large vacuoles occur in the cytoplasm of the endothelial cell. The markers represent 1 μ.

Fig. 4. A magnified portion of Fig. 2. Running from top to bottom in the centre of the illustration is the basement membrane showing the epithelial cell processes ending abruptly on the left surface and a thin coating of endothelial cell cytoplasm on the right surface. There are no pores in the membrane. The distance between markers is 1 μ.

The nuclei of the endothelial cells projected into the lumina of the glomerular loops, and although commonly oval in appearance, some were "kidney"-shaped or irregular in outline due to compression by circular spaces in the adjacent cytoplasm (Fig. 2). The amount of cytoplasm visible in individual capillary loops varied a great deal. In some, an extremely thin layer lay against the basement membrane, distinguishable only by the different density of the two structures, but at irregular intervals it projected as flattened or polypoid masses into the capillary lumina or expanded to surround adjacent endothelial nuclei. Vacuoles were common in the cytoplasm of both epithelial and endothelial cells but were more numerous and larger in the latter and in sections showing cellular fragmentation, groups of small closely-packed vacuoles were seen in the cytoplasm of the endothelial cells. The significance of this will be discussed in a later section. Some lumina were partly bridged by thin lines surrounding large circular spaces 2 to 3μ in diameter, and these were more numerous in badly preserved material.

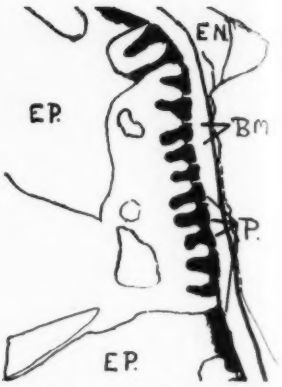

Fig. 5. A diagram of the central portion of Fig. 4, showing the tooth-like processes (P) surrounded by a membrane which separates them from the basement membrane (B). Parts of epithelial cell cytoplasm (EP) and endothelium (EN) are shown.

DISCUSSION.

A somewhat clearer conception of the morphology of the glomerulus has followed electron microscopic studies. However, with the great increase in magnification, it is not surprising that various structural interpretations have resulted from initial observations on the glomerulus.

The presence of a finite basement membrane between the capillary endothelium and the visceral layer of Bowman's epithelial cells is shown in most studies (Pease and Baker, 1950; Dalton, 1951; Oberling, Gautier and Bernhard, 1951), and the different electron density of the three major glomerular components is seen in the present investigation.

No support has been found for the suggestion by Rinehart *et al.* (1953) that the basement membrane is traversed by projections from the epithelial cells, and the small closely-packed vacuoles in the cytoplasm of the endothelial cells do not occur in the basement membrane proper. It is suggested that the "honeycombed" endothelium of Oberling, Gautier and Bernhard (1951), and the "mucoid" projections of Rinehart *et al.* (1953), may represent degenerative changes in the endothelium.

The complex system of projections and arcades on the external surface of the basement membrane appears to be derived from the cytoplasm of the visceral epithelial cells. In many cases this is quite obvious (Fig. 2). In others, rows of processes of the same density and morphology, each surrounded by a definite membrane, are applied to the outer surface of the basement membrane (Figs. 4 and 5), but do not penetrate it. Pease and Baker (1950) suggest that the function of these structures may be a mechanical one, giving support and strength to the basement membrane. As the projections do not arise from the basement membrane and the marked increase in surface area produced by the interdigitations of the processes is apparent, they may possibly have a more active rôle connected with the production of glomerular filtrate.

SUMMARY.

The glomerular structure of the normal adult mouse kidney has been studied by means of the electron microscope.

The basement membrane of the glomerulus appeared as a definite layer which differed in density from the cytoplasm of adjacent epithelial and endothelial cells.

No pores or penetration of the membrane by projections of the epithelial cells could be seen, and no processes appeared to originate from it.

Vacuoles, which occurred to a greater extent in the cytoplasm of the endothelial cells than in the epithelial cells, were common and probably represented autolytic or fixation artefacts.

Acknowledgments. I am indebted to Professor J. S. Robertson for technical advice and assistance during this study, and to Dr. S. G. Tomlin and Mr. Ian McLean, B.Sc., of the Department of Physics, University of Adelaide, for much assistance and advice with the electron microscope.

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THE CHANGES IN CARDIAC OUTPUT, RIGHT ATRIAL PRESSURE AND BLOOD VOLUME IN HAEMORRHAGIC ANAEMIAS IN UNANAESTHETIZED RABBITS

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In severe anaemias it seems to be clearly established that there is a rise in cardiac output, both in man at rest (Sharpey-Schafer, 1945; Brannon, Merrill, Warren and Stead, 1945) and in dogs (Blalock and Harrison, 1927). However, associated circulatory changes have been less well defined. Thus, the results of determinations of mean right atrial pressures in anaemia are conflicting, Sharpey-Schafer (1945) reporting a marked rise in severe anaemias in man, while Brannon *et al.*, in a comparable group of anaemic patients, could find no such change. Similarly, a variety of changes have been reported in the blood volume in anaemias in man. Plesch (1922), Hartwich and May (1926), and Sjöstrand (1949) reported an expansion of blood volume. Keith, Rowntree and Geraghty (1915) found little change, while others (Bennett, Dow, Lander and Samson Wright, 1938; Gibson, Harris and Swigert, 1939; McMichael, Sharpey-Schafer, Mollison and Vaughan, 1943; Sjöstrand, 1949) have found a reduction in blood volume of varying degree. Probable factors accounting for these diverse results have been the multiple techniques used for determining the blood volume and the varied aetiology of the anaemias considered within many series.

The experiments reported here deal with circulatory changes occurring in haemorrhagic anaemias in resting unanaesthetized rabbits. The relationships of right atrial pressure, cardiac output and blood volume to the degree of anaemia were investigated.

METHODS.

Rabbits, mostly albino, weighing 1·3 to 2·6 kg., were used for all experiments. For each group of experiments the mean body weights were approximately the same.

Production of anaemia. The rabbits were bled 14 ml./kg. daily from the lateral ear vein. At no stage did this haemorrhage seriously disturb the animal. Rabbits were not bled more than four times, because after this red cell regeneration compensated for the daily loss and prevented a further fall in haemoglobin concentration. Cardiac outputs, right atrial pressures and blood pressure estimations were made 24 to 72 hours after the last haemorrhage.

[1]This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

Anaesthesia. One half p.c. procaine hydrochloride injected locally, together with intravenous, aqueous, thiopentone sodium (20 mg./ml.) was found to be the most satisfactory anaesthetic for inserting the tracheotomy tube and cardiac catheters. The usual dose of thiopentone sodium for the anaemic animals was 8 to 10 mg./kg. Even with this minimal anaesthesia, residual effects were often still evident up to 3 hours after the injection. In all anaemic animals, therefore, cardiac output determinations were made 5 to 6 hours after giving the anaesthetic.

Cardiac output determinations. Cardiac outputs were determined by the direct Fick method, as previously described by Courtice and Korner (1952). Most animals were suspended in the head-down position 5 to 10° from the horizontal. A small series of cardiac output estimations were also made on animals lying quietly on their left side in the rabbit bag.

Right atrial pressures. About half of the pressure estimations were made through the catheter inserted for the cardiac output determinations. In the remainder, a polyethylene catheter was passed down the central vein of the ear into the right atrium as described by Armin, Grant, Pels and Reeve (1952). The position of the catheter tip was checked post-mortem in all cardiac output determinations. Except when the tip was in the atrium, a sample of 1 ml. of blood could not be drawn back readily into a syringe. This was used as a criterion for the correct positioning of the catheter tip. The method of recording atrial pressure was a modification of that described by Burch and Winsor (1943). The zero point of reference was the upper end of the sternum in the suspended animal.

Systemic blood pressure. During cardiac output estimations mean carotid arterial pressures were measured through the carotid catheter with a mercury manometer. A series of systolic blood pressure determinations were also made in the central artery of the ear by the method of Grant and Rothschild (1934). The oxygen saturation of blood samples was determined with the modified Haldane blood gas analysis apparatus (Courtice and Douglas, 1947). Oxygen capacities could not be determined in the samples from severely anaemic animals, however, because of the rapid auto-oxidation which occurs in markedly lipaemic plasma on adding potassium ferricyanide (Courtice and Douglas, 1947). In these samples the oxygen capacity was determined indirectly by repeated haemoglobin estimations using a calibrated M.R.C. grey wedge photometer. Expired air samples were analysed with the Haldane gas analysis apparatus.

Blood volume. Evans Blue (T1824) was used for blood volume determinations. Blood samples were taken before the administration of dye and at intervals of 15 and 30 minutes after injection into the lateral ear vein or into the atrial catheter. In the first 34 blood volume estimations an additional sample was taken at 22 minutes. Aqueous T1824 (1·6 ml. of a solution containing 600 mg./litre) was injected and the dye extracted from plasma samples with acid *n*-butanol (Harington, Pochin and Squire, 1940). When using this method of extraction 9 ml. of blood were required for a blood volume estimation. Because of this, such determinations were made only when the animal was bled to produce anaemia, or, as a terminal estimation. In the majority of blood volume determinations 2 ml. of T1824 (900 mg./litre) were injected and the dye extracted by a modification of the method of Allen (1951). For this only 0·5 ml. plasma was needed for each sample and so the whole blood volume estimation required only about 4·5 ml. of blood. Extraction of the dye from plasma was essential in these animals because of the gross lipaemia which developed after the animals were bled. Optical density determinations were made on butanol extracts of dye with a Hilger "Spekker" photoelectric absorptiometer, and after extraction by Allen's method, with a Beckman quartz spectrophotometer at a wavelength of 620 mμ. Plasma dye concentrations were then determined from standard calibration curves prepared for each solution made up. The theoretical dye concentration at the instant of injection was determined by linear extrapolation of the determined points. Haematocrits were done, always at least in triplicate, in glass tubes 1 mm. diameter with an approximately 10 cm. column. These

were spun for 55 minutes at 3,000 rev./min. with a standard 15 cm. radius centrifuge head. No correction was made for trapped plasma (Chaplin and Mollison, 1952).

In expressing mean values in this paper, the standard error of the mean is stated in all instances.

RESULTS.

Cardiac output.

The cardiac output determinations in 25 rabbits suspended in the rabbit sling, with haemoglobin concentrations varying from 15·7 to 3·5 gm./100 ml., are summarized in Fig. 1. Recumbent animals in which the cardiac output was determined are not included in the figure because of the differences caused by the posture (see Table 1). As the haemoglobin concentration fell from 15·7 to

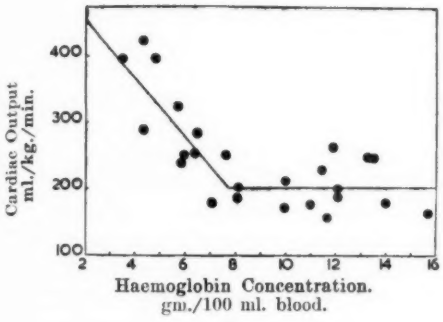

Fig. 1.

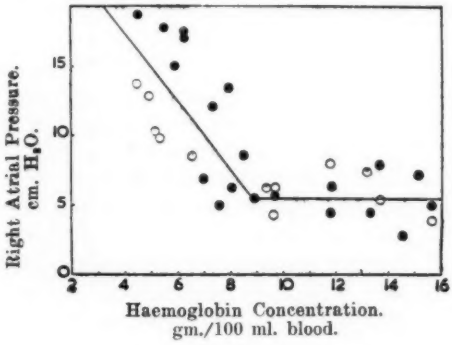

Fig. 2.

Fig. 1. The relationship between haemoglobin concentration and cardiac output in graded haemorrhagic anaemias in rabibts. The oblique line represents the statistically significant regression of cardiac output on haemoglobin concentration below a haemoglobin level of 7·5 gm./100 ml. blood.

Fig. 2. The relationship between haemoglobin concentration and right atrial pressure in haemorrhagic anaemias in rabbits.

O = Atrial pressure determinations simultaneous with cardiac output determination.
• = Atrial pressure determination with catheter inserted via central ear vein.

The oblique line represents the statistically significant regression of right atrial pressure on haemoglobin concentration below a haemoglobin level of 7·5 gm./100 ml. blood.

7·5 gm./100 ml., there was no significant change in cardiac output, the mean value being 203 ± 10·3 ml./kg./min. Below this haemoglobin level the cardiac output rose to approximately 400 ml./kg./min. at a haemoglobin concentration of 3·5 gm./100 ml. The regression of the mean cardiac output on the fall of the haemoglobin level below 7·5 gm./100 ml. was statistically significant ($p = 0·001$). Changes of a similar order were observed in 7 recumbent animals in which the cardiac output was determined. As these changes in minute volume

in anaemia were unaccompanied by any significant change in heart rate, it may be concluded that the rise in cardiac output was due to a rise in stroke volume. In order to determine whether the heart rate changes were masked by the suspension of the animal and other factors involved in cardiac output determinations, pulse rates were measured in a separate group of 20 animals during the bleeding programme, in the ear with a Grant capsule in the undisturbed animal in a rabbit box. Pulse rates were slightly lower in this group than in the cardiac output series, but no rise with progressive anaemia was noted either in mean values or in individual animals.

Right atrial pressures.

In Fig. 2 the right atrial pressure has been plotted against the haemoglobin concentration. The right atrial pressure did not change significantly until the haemoglobin level fell to 7·5 gm./100 ml., the mean value over this range being $5{\cdot}7 \pm 0{\cdot}4$ cm. H_2O. With further progressive fall in the haemoglobin level, the right atrial pressure rose abruptly to approximately 17 cm. H_2O at a haemoglobin concentration of 4 gm./100 ml. The regression of the rise in atrial pressure on the drop in the haemoglobin level was statistically significant ($p = 0{\cdot}01$): The results in Fig. 2 are taken from two groups of animals: one series in which the atrial pressure was measured at the time of cardiac output determination, whilst in the other pressure readings were made with a polyethylene catheter passed down the central ear vein into the atrium. There was no significant difference between the two groups. Changes in intrapleural pressures probably contributed little to the mean rise in right atrial pressure for respiratory rates, and minute ventilation did not alter significantly as the haemoglobin level fell.

Systemic blood pressure.

In the group of animals in which cardiac outputs were estimated, the mean carotid blood pressure fell progressively with the haemoglobin concentration. This relationship may be summarized by the following regression equation:

$$\text{B.P.} = 89{\cdot}50 + 2{\cdot}99\ (\text{Hb} - 8{\cdot}93)$$

where B.P. = mean carotid blood pressure, mm. Hg.

Hb = haemoglobin in gm./100 ml.

and p for significance of regression coefficient from zero = 0·001.

This equation indicates that for a fall in the haemoglobin concentration of 1 gm./100 ml. blood, the mean systemic blood pressure fell by 2·99 mm. Hg. A similar relationship was found in a series of 67 observations made on systolic blood pressures in the central ear artery, using the Grant capsule method. The regression coefficients, expressing the relation of arterial pressure to haemoglobin concentration, did not differ significantly in the two series.

Gaseous exchange.

Pulmonary ventilation per minute, oxygen consumption per minute, respiratory rate and the respiratory quotient of expired air did not change significantly with increasing anaemia. The mean oxygen consumption was $12{\cdot}31 \pm 0{\cdot}59$ ml./kg./min. Changes in gaseous exchange between the blood and tissues were similar to those reported in man. The percentage oxygen saturation of arterial blood did not change significantly. There were four severely anaemic animals in which the percentage oxygen saturation fell below 92 p.c., but the remaining nine anaemic animals in this range had arterial saturations from 92 to 98 p.c. The percentage oxygen saturation of mixed venous blood fell progressively with the haemoglobin concentration, and, as expected, percentage oxygen utilization by the tissues rose progressively from the control value as described by the linear regression:

$$U = 50{\cdot}0 - 3{\cdot}80\ (Hb - 8{\cdot}93)$$

where U = percentage oxygen utilization

Hb = haemoglobin in gm./100 ml.

p for significance of regression coefficient from zero = 0·001.

This means that for a reduction in haemoglobin concentration of 1 gm./100 ml. the percentage oxygen utilization increased 3·80 p.c. These findings do not differ significantly from those in man (Sharpey-Schafer, 1945; Richards and Strauss, 1928) over the same range. At haemoglobin levels below 3·5 gm./100 ml., however, the percentage oxygen utilization by the tissues rises much more rapidly in man (Sharpey-Schafer).

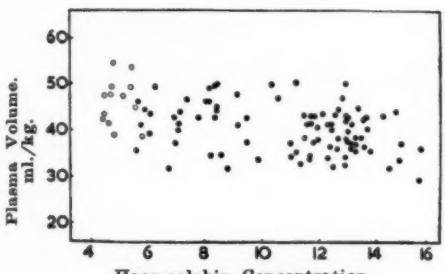

Fig. 3.

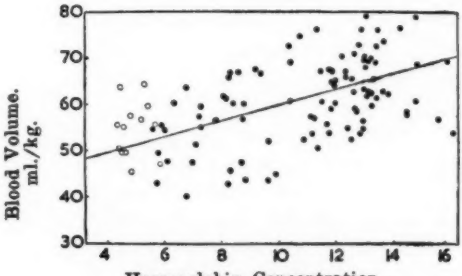

Fig. 4.

Fig. 3. The relationship between haemoglobin concentration and plasma volume in haemorrhagic anaemias in rabbits. The mean of the plasma volumes in those animals bled 3 or 4 times, represented by open circles, was significantly higher than the mean of the remainder.

Fig. 4. The relationship between haemoglobin concentration and blood volume in haemorrhagic anaemias in rabbits. The line represents the statistically significant linear regression for these points. The blood volumes in those animals bled 3 or 4 times, represented by open circles, did not deviate significantly from the regression line.

Blood volume.

In all animals, except those used for control cardiac output estimations, blood volumes were determined at least twice, one of them always before removal of any blood. In Fig. 3 plasma volumes/kg. are plotted against haemoglobin concentration. In all, 105 plasma volume determinations were made. For the whole series, over a range of haemoglobin levels 15·7 to 3·5 gm./100 ml., there was no significant change in plasma volume, the mean value being 40·7 ± 0·7 ml./kg. In 13 animals with a haemoglobin below 5·8 gm./100 ml., which had been bled three or four times, the mean plasma volume was 46·1 ± 1·4 ml./kg. This was significantly different from the mean of the whole group. The corresponding blood volumes were not, however, increased significantly above the expected value.

The change in blood volume with a falling haemoglobin concentration from 15·7 to 3·5 gm./100 ml. is shown in Fig. 4 by the linear regression whose equation is:

$$B.V. = 61{\cdot}69 + 1{\cdot}79\ (Hb - 12{\cdot}01)$$

where B.V. = blood volume/kg. in ml.

Hb = haemoglobin concentration in gm./100 ml. blood.

p for significance of regression coefficient from zero = 0·001.

This means that for a fall in haemoglobin concentration of 1 gm./100 ml., the blood volume fell 1·79 ml./kg.

To determine if the above changes in plasma and blood volume were influenced by the preceding haemorrhages, blood volume estimations were made on 9 animals during the bleeding programme and in the subsequent phase of recovery. Three animals were bled on 1 day, 3 on 2 successive days and 3 on 4 successive days. Four or five blood volume determinations were made over the recovery period of 14 to 16 days. The amount of blood removed for each determination was 4·5 ml. and probably did not significantly affect the results. The changes in the period of recovery were essentially those described for the above larger series, except that in the few plasma volumes determined when the

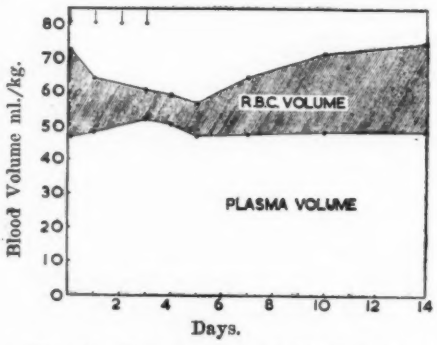

Fig. 5. Changes in total blood volume, plasma volume and red cell volume produced by daily bleeding 14 ml./kg. on 4 successive days and followed through the phase of recovery. Each haemorrhage is indicated by an arrow ↓ . At the end of the fourth day haematocrit was 14·4 p.c. (haemoglobin 4·7 gm./100 ml.). Each point represents the mean of 3 determinations.

haemoglobin concentration was below 5·8 gm./100 ml., the expansion was not statistically significant. Fig. 5 illustrates the average changes in 3 animals in total plasma and red cell volumes during the periods of bleeding and recovery. In the whole series of 9 animals, plasma volumes were significantly higher than in the main series. The mean of the control plasma volumes was 43·6 ± 1·6 ml./kg. The variance ratios were not significantly different.

In 61 normal rabbits, the mean plasma volume per kilogram was 39·6 ± 0·6 ml., agreeing closely with the figures of Courtice and Gunton (1949) and Armin, Grant, Pels and Reeve (1952). Mean total blood volume per kilogram was 63·8 ± 1·0 ml. The means of comparable groups of plasma volumes in which the dye extraction methods of Harington, Pochin *et al.*, and Allen, respectively, were used, did not differ significantly.

TABLE 1.

Changes in cardiac output and right atrial pressure on expanding blood volume in anaemic animals to approximately normal values by transfusion with blood of the same haemoglobin concentration. "Control" blood volume in the table refers to the blood volume estimation done on the animal prior to producing any anaemia. The blood volume after transfusion was calculated from the blood volume before transfusion and the subsequent change in haemoglobin concentration.

Haemoglobin concentration gm./100 ml.	Blood volume ml./kg.			Cardiac output ml./kg./min.		Right atrial pressure cm. H_2O	
	Control	Before transfusion	After transfusion	Before transfusion	After transfusion	Before transfusion	After transfusion
(a) *Moderately anaemic rabbits:*							
12·9	77·0	77·0	88·6	275	343	7·5	9·0
9·9	72·0	72·0	86·4	340	317	4·5	6·5
9·5	60·8	44·7	56·6	250	190	6·0	8·0
9·6	76·7	60·4	74·3	321	300	6·0	7·5
Mean	Increase in B.V./kg. with transfusion 12·8 ± 0·9			297 ± 30·0	288 ± 33·7	6·0 ± 0·6	7·8 ± 0·5
(b) *Severely anaemic rabbits:*							
5·2	58·4	45·7	61·1	414	500	10·5	14·0
5·2	64·8	56·5	68·8	583	594	10·5	14·0
4·7	76·0	54·5	59·5	625	396	13·0	17·5
Mean	Increase in B.V./kg. with transfusion 10·9 ± 3·1			541 ± 49·8	497 ± 57·1	11·3 ± 0·8	15·2 ± 1·2

Thus, in haemorrhagic anaemias in rabbits, the plasma volume remained constant and the reduction in total blood volume was the result of the change in the red cell volume.

Circulatory effects of expanding the blood volume.

A series of seven animals was used to determine the circulatory effects of increasing the low blood volume in anaemic animals to normal levels without changing the haemoglobin concentration. Interest was directed particularly towards cardiac function in the anaemic animals under such a stress. The

animals were prepared in the usual way for cardiac output determinations, with the addition of a third polyethylene catheter in the external jugular vein for transfusing the animal. Also, because the experiments were prolonged, except for the initial right atrial pressure reading, the animal was not suspended, but lay quietly on its left side. Control cardiac output and blood volume determinations were made and the transfusion begun. Over a period of 2 hours 15 ml./kg. of blood, with a haemoglobin level slightly below that of the animal, was given. Mean systemic and right atrial pressures were recorded during the experiment and a second cardiac output estimated just before terminating the transfusion.

The main circulatory changes resulting from this increase in blood volume are summarized in Table 1. There was no significant change in haemoglobin concentration, mean carotid blood pressure, heart rate, or oxygen consumption during the experiments. The control cardiac outputs and oxygen consumptions were significantly higher in the recumbent animals than in suspended animals over the whole range of these anaemias. Postural effects of a similar type have been reported in normal human subjects (Donald, Bishop, Cumming and Wade, 1953). The mean oxygen consumption was $15{\cdot}16 \pm 0{\cdot}88$ ml./kg./min.

At the end of the experiment about 80 p.c. of the transfused fluid was retained by both the groups of animals described in the table. The significantly greater rise of the right atrial pressure in the severely anaemic group of animals was unaccompanied by a corresponding rise in cardiac output. These findings suggest that the heart in the severely anaemic animals was still competent, but that its capacity to increase further its output was very limited and loading greater than that used would probably precipitate cardiac failure.

DISCUSSION.

Circulatory changes.

Post haemorrhagic anaemia in unanaesthetized rabbits was accompanied by circulatory changes similar to those previously reported in man. The haemoglobin level at which a marked rise in cardiac output occurred and the order of the rise (about 100 p.c. of the control value when haemoglobin concentration fell to 4 gm./100 ml.) agree with similar findings in man (Sharpey-Schafer, 1945; Brannon *et al.*, 1945; Dautrebande, 1925). In Blalock and Harrison's work on dogs this rise occurred at a higher haemoglobin level and was much more variable than in this or reported human series. In man, an increase in heart rate is a contributing factor to the rise in cardiac output, but in rabbits was apparently not significant, the stroke volume rising concomitantly with cardiac output.

Right atrial pressure determinations in man in "high output failure," have been conflicting. Brannon *et al.* (1945) found no significant change in central venous pressure in patients with severe anaemia. On the other hand Sharpey-

Schafer found a marked rise in a similar human series. The sharp rise in right atrial pressure in rabbits when the haemoglobin concentration fell below 7·5 gm./100 ml. agrees with the latter's findings in man. In these animals the coincident rise in cardiac output and right atrial pressure suggests that the heart was competent at rest, even in the most severe of these experimental anaemias. Further, moderate loading of the heart by transfusion did not precipitate frank failure, as judged by the fact that the cardiac output remained unchanged while the right atrial pressure simultaneously rose. However, the absence of a significant rise in cardiac output under such circumstances, indicative of the inability of the heart to increase further its output, suggests that the myocardium was approaching its point of failure.

Apparently in the resting anaemic animal, "back pressure" effects of a failing heart were not the primary factors in elevating the right atrial pressure. More probably, as Sharpey-Schafer and McMichael have suggested, this rise was the immediate cause of the rise in cardiac output. Further, these findings in the rabbit support McMichael's (1949) suggestion that this rise in central venous pressure "must be the last resource of the compensating mechanisms."

How such a rise in right atrial pressure is produced is not clear. With a competent heart the main factors influencing it are (1) the venous blood volume, and (2) the volume elasticity coefficient of the venous vascular system which may be altered rapidly by changes in the tone of the muscular wall. Landis (1950) estimates that approximately 65 p.c. of the total blood volume is normally contained in the venous system. A 20 to 25 p.c. fall in blood volume, such as occurred in the severely anaemic animals, would probably be reflected mostly in this distensible low pressure system. Regional redistribution of blood may also have occurred, with displacement of blood into the venous system, such as has been suggested in acute anoxia and during noradrenaline infusions (Courtice and Korner, 1952; Korner, 1953), but because of the marked reduction in blood volume could not alone account for the rise in central venous pressure. Thus, the important factor is apparently an active reduction in the total venous vascular capacity, caused by local or general vasomotor changes. That such a reduction in the volume of the venous vascular system did occur is supported by the findings in the transfusion experiments. With transfusions of equal volume, other circulatory changes being minimal, the right atrial pressure rose 4 cm. H_2O in severely anaemic rabbits and only 1·8 cm. H_2O in the controls. This suggests that in the anaemic animals the tension in the wall of the venous system more nearly approached its elastic limit.

Little work has been done on the possible regional distribution of these vasomotor changes in anaemias. However, Bradley and Bradley (1947) reported a marked fall in renal blood flow, and Stewart, Crane and Deitrick (1937) and Abramson, Fierst and Flachs (1943) have reported reduced blood flow in the hand of anaemic patients. The latter, however, found increased blood flow in the forearm. This local arteriolar vasoconstriction, reducing blood flow, would

also reflexly reduce the capacity of the local drainage veins, as has been demonstrated by Fleisch (1931).

Such findings suggest that the impairment of oxygen transport and diminution in blood volume in anaemias are met by increasing the total blood flow through a vascular circuit smaller than in the normal animal.

Changes in total peripheral resistance in anaemic rabbits were similar to those reported in man, a slight but significant fall occurring as the haemoglobin level fell to 7·5 gm./100 ml., and below this a further sharp fall coincident with the rising cardiac output. These have been considered to indicate a generalized vasodilation, but in view of other circulatory changes in anaemia one might expect such vasomotor changes to be injurious to the animal. Green, Lewis, Nickerson and Heller (1944) have clearly shown the lack of correlation between changes in peripheral resistance and vasomotor tone when either mean pressure and/or flow rate alters markedly. Moreover, in anaemias there is the further variable of blood viscosity affecting peripheral resistance. Thus, interpretation of this marked fall in total peripheral resistance, associated with a marked rise in cardiac output, as indicating generalized arteriolar vasodilatation is hazardous, and in view of the other haemodynamic changes, unlikely.

Blood volume changes.

The constancy of the plasma volumes in this series of 145 determinations in rabbits, over a wide range of haemoglobin concentrations, is striking, particularly in view of some of the previously reported changes in anaemias in other species. Owing to the inherent variation in the normal blood volume in man and other species, a moderately large series of observations is necessary to detect significant changes in pathological states. Much previously reported work has been done in quite small groups of anaemic patients, with added variables such as the aetiology of the anaemia, the general condition of the patient and the method of blood volume determination.

Bock (1921) and Bennett *et al.* (1938), using the Congo red dye method, found a constant plasma volume in haemorrhagic anaemias in man. However, in most reports on human anaemias an expansion of the plasma volume is described. Gibson *et al.* (1939), using the T1824 method, without plasma extraction, found an expansion of about 30 p.c. in severely anaemic patients. Because of this, the total blood volume was only slightly less than the normal value. However, in their series of haemorrhagic anaemias, serial determinations were rarely performed, the measured volumes being compared with the expected blood volume for the height of the patient. Sjöstrand (1949), using the carbon monoxide method, reported similar findings in a small series of haemorrhagic anaemias in man, although in other human anaemias he reported plasma volume expansion sufficient to increase the blood volume.

On the other hand, McMichael, Sharpey-Schafer, Mollison and Vaughan (1943) and Sharpey-Schafer (1945), using the red cell transfusion method of Hill (1941), found extremely low blood volumes in a series of haemorrhagic anaemias in man. This method, as was pointed out by these workers, is subject to serious error. In view of these findings, Sharpey-Schafer restated the previous suggestion of Bock that the "reduction in blood volume" in severe anaemias "is a more active process than a simple decrease in the number of red cells," implying a reduction of the plasma volume in an attempt to maintain an adequate haemoglobin concentration. Apparently, since the plasma volume does not change, no such compensation occurs in the anaemic rabbit.

The slight expansion of the plasma volume when the haemoglobin level fell below 6 gm./100 ml. was probably of little physiological importance. It may be related to the prior haemorrhage, as it occurred only in animals bled three or four times. However, this has not yet been investigated.

SUMMARY.

Circulatory effects of graded haemorrhagic anaemia were investigated in unanaesthetized rabbits.

The cardiac output, determined by the direct Fick method, and the right atrial pressure did not change significantly until the haemoglobin level fell below 7·5 gm./100 ml. blood. Both then rose abruptly until, at a haemoglobin concentration of 4 gm./100 ml., the cardiac output was doubled and the right atrial pressure had risen from a mean value of 5·2 cm. H_2O to 17 cm. H_2O. As the degree of anaemia increased the pulmonary ventilation, oxygen consumption and respiratory quotient remained unchanged, while the percentage oxygen utilization by the tissues rose progressively, and the mean systemic blood pressure fell slightly but significantly.

The plasma volume, determined by the T1824 method, in anaemic animals remained constant until the haemoglobin concentration fell below 6 gm./100 ml., when there was a slight expansion. Thus, the blood volume was reduced in these anaemias and varied directly with the red cell volume.

Expanding the total blood volume in anaemic animals to the normal value, by transfusion with blood of the same haemoglobin concentration, did not precipitate frank cardiac failure, but demonstrated the very limited capacity of the heart to increase further its output. It was concluded that the rise in right atrial pressure was not primarily caused by heart failure, but was active, compensatory and possibly the result of widespread vasoconstriction.

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STUDIES OF COXSACKIE VIRUSES

1. COMPARISON OF AGE-SUSCEPTIBILITY RELATIONSHIPS IN MICE

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In a previous publication (Howes, 1952) an unusual relationship between age and susceptibility of mice, bred at this Institute, to infection with two Coxsackie virus strains, Connecticut 5 and Adelaide 1, was described. These observations were extended to a further six Group A and three Group B strains in the hope that age susceptibility relationships (A.S.R.'s) might serve to differentiate between the two groups and individual members of each group. Results of these experiments are presented in this paper.

North American strains examined were Dalldorf 2, Dalldorf 3, Easton 2, Easton 14, Texas 1, Ohio R, and Nancy. These strains were very kindly supplied by Dr. J. L. Melnick of the Section of Preventive Medicine, Yale School of Medicine, to Dr. J. A. R. Miles of this Institute. A further two South Australian strains, Woodside 1 (Group A, Type 2) and Adelaide 2 (Group B, Type 3), were also examined. These strains were isolated from cases diagnosed as pleurodynia.

Methods.

Virus suspensions. These were prepared as 20 p.c. w/v suspensions of infected muscle harvested from two to three days after inoculation of 0–2 days old mice. Suspensions were made in 10 p.c. inactivated rabbit serum-saline containing penicillin and streptomycin at concentrations of 500 units per ml. After overnight extraction at + 4° C., and centrifugation in an angle centrifuge at 1,500 r.p.m. for 10 minutes, virus suspensions were dispensed in 1 ml. amounts and stored in a dry-ice cabinet until used.

Virus dilutions. For each titration, two 1 ml. lots of the same virus pool were mixed, and tenfold dilutions of muscle were prepared in final volumes of 2·5 ml. Ten per cent. rabbit serum-saline plus penicillin and streptomycin (as above) was used as the diluent.

Mice. The mice used in these experiments were from an inbred strain of albino mice maintained at this Institute. Ages of suckling mice were known to within ± 12 hours, and of newly-weaned mice to within ± 36 hours. The size of mice in each age group was kept within limits as small as possible by discarding poorly developed mice and large mice of small litters. In each age group all suckling mice were pooled and redistributed to the does.

Selection of age groups. Age groups used in the present series of experiments were 0–1, 3–4, 7–8, 9–10, 12–13, 16–17 and 21–24 days. Older mice were occasionally used. The age groups chosen were unevenly spaced so that the period of relative insusceptibility found with

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the Connecticut 5 and Adelaide 1 strains, most marked in 9–10 days old mice, might be adequately covered.

Bulk staining of pancreatic tissue. The pancreas of each survivor of infection with group B strains was dissected out, fixed overnight in Bouin's fluid, and stained for 1–2 hours in a saturated solution of Scharlach R in equal parts of 70 p.c. ethyl alcohol and acetone. After briefly washing in 70 p.c. alcohol, each pancreas was examined for the scarlet areas indicating replacement of pancreatic acinar tissue by fat.

General procedure. Susceptibility of mice of different ages was assessed by titrating the one pool of virus in different age groups. The intraperitoneal route and an inoculum of 0·05 ml. of virus dilution was used throughout.

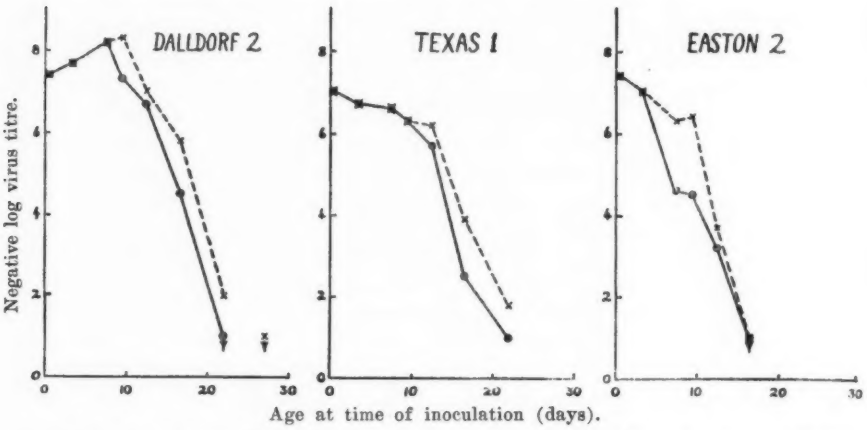

Fig. 1. Age-susceptibility relationships of Group A Coxsackie virus strains. Upper interrupted line = ID_{50} titres. Lower continuous line = LD_{50} titres.

For each virus strain titrations were carried out in at least seven age groups using 5 or more suckling mice (usually 6 or 7), and 5 weaned mice per dilution. From 4 to 9 virus dilutions were tested for each age group, the titrations being repeated if necessary.

In most cases titrations were not completed in the one experiment, but, depending on the supply of mice of a suitable age, were spread over two or three experiments.

Suckling mice were inspected daily for 21 days and then killed. Weaned mice were inspected daily for 21 days for group A strains, and 28 days for group B strains, and then killed.

The titres of virus in mice of different age groups must be influenced to some extent by the use of a constant inoculum in mice varying considerably in weight. However, the underestimation of titre in older mice, due to increased weight, would be only slightly more than 1 log over the age range considered in these experiments, and does not invalidate the conclusions drawn from the results.

Calculations of virus titres. The 50 p.c. lethal dose of virus (LD_{50}), and the 50 p.c. infectious dose (ID_{50}) were calculated by the method of Reed and Muench (1938).

LD_{50} titres were calculated from the number of deaths which occurred during the period of observation.

ID_{50} titres were calculated by adding to the number of deaths at each dilution the number of survivors which had shown paralysis or unequivocal muscle weakness; or, alter-

natively, the number of survivors in which, by bulk staining, pancreatic lesions were shown to have occurred. Unequivocal muscle weakness was, for the purpose of these experiments, defined as definite muscle weakness as shown by restricted limb movement observed on two successive days. Deaths which occurred during the first 48 hours after inoculation were regarded as nonspecific since incubation periods of 48 hours or less have only been seen when very high concentrations of virus have been used.

EXPERIMENTAL.

Group A strains.

Age and susceptibility to lethal infection. Results of titrations of five American serological types and one South Australian strain (Woodside, Type 2) are presented in Table 1. The A.S.R.'s were similar in type in all cases although differences between strains were apparent with respect to the age at which a reduction in susceptibility was first seen. Results for three of these strains are presented graphically in Fig. 1.

For all but one strain (E2), susceptibility to lethal infection fell but slightly (less than one log) or not at all during the first ten days after birth. This period of little change in susceptibility was followed by a decline in susceptibility until, in 21–24 days old mice, one strain (Dalldorf 3) did not infect, four gave less than 50 p.c. deaths when inoculated with a 10 p.c. suspension, and only one (Texas 1) caused over 50 p.c. deaths. Susceptibility appears therefore to be lost soon after an age of 3 weeks has been reached.

TABLE 1.

Age susceptibility relationships for Group A strains of "C" virus.

LD_{50} and ID_{50} titres of virus pools in mice of different age groups.

Age of mice days)	Easton 2 (Type 1) (1)	(2)	Dalldorf 2 (Type 2)	Dalldorf 3 (Type 3)	Texas 1 (Type 4)	Easton 14 (Type 5)	Woodside 1 (Type 2)
0–1	7·4*	6·8	7·4	7·2	7·0	7·4	7·5
	7·4*	6·8	7·4	7·2	7·0	7·4	7·5
3–4	7·0	6·8	7·7	7·1	6·7	7·7	7·2
	7·0	7·1	7·7	7·1	6·7	7·7	7·2
7–8	4·6	5·6	8·2	6·9	6·6	7·3	7·3
	6·3	6·1	8·2	6·9	6·6	7·3	7·3
9–10	4·5	4·0	7·3**	6·6	6·3	6·7	7·6
	6·4	5·4	8·2	6·7	6·3	6·7	7·6
12–13	3·2	4·2	6·7	5·0	5·7	6·2	7·2
	3·7	5·3	7·0	5·4	6·2	6·3	7·4
16–17	<1	1·0	4·5	3·2	2·5	4·7	4·2
	<1	1·8	5·8	3·2	3·9	4·7	6·1
21–24	<1	<1	<1	<1	1·0	<1	<1
	<1	<1	2·0	<1	1·8	1·4	2·2
33–35	—	—	<1	—	—	—	—
			<1				

* Upper and lower values represent LD_{50} and ID_{50} titres respectively.

** 10–11 days old mice.

No signs of infection were seen with the Dalldorf 3 strain in 21–24 days old mice, but since this strain was, in other age groups, comparable in its behaviour to those strains which produced infection in 21–24 days old mice, and since large doses of virus were required to produce infection with those strains, Dalldorf 3 was not considered to differ from them in any significant way.

However, the Easton 2 strain produced infection in only one of the 21–24 days old mice inoculated with a 10 p.c. suspension, and titres in all other age groups with the exception of 0–1 and 3–4 days old mice were consistently lower than titres obtained with other strains, suggesting a real difference between the E2 strain and the other group A strains investigated.

Occurrence of sub-lethal infection. For the virus strains other than Easton 2, no mice up to 7–8 days old which had shown paralysis or unequivocal muscle weakness, recovered. Beyond this point, further increase in age was attended by a marked increase in the number of mice recovering from obvious infection (Fig. 2). With the Easton 2 strain, the earlier reduction in susceptibility was associated with an earlier occurrence of sub-lethal infections, this association supporting the view that in its age susceptibility relationship this strain differs from other group A strains.

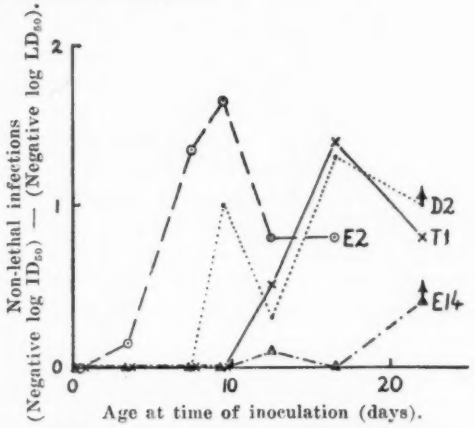

Fig. 2. Occurrence of non-lethal infections with Group A strains, Easton 2, Dalldorf 2, Easton 14 and Texas 1 in relation to age.

Nature of disease in suckling and weaned mice. Paralysis or muscle weakness was produced by all virus strains and was common to infected mice of all age groups, although the symptoms produced by the Easton 2 strain differed to some extent from those produced by other strains. The marked ataxia and tremors observed in infection with some group B strains were not seen, and similarly, cachexia was not so prominent a feature. Modification in the severity of effects of the muscle lesion was seen as the age of mice increased. After onset of the disease in newborn mice, muscle weakness as a rule progressed rapidly to a complete flaccid paralysis of the hind limbs, often accompanied by evidence of weakness or paralysis of one or both forelegs. Even at dilutions near titration end-points death usually occurred within three days of signs of infection first having been observed.

With increase in age the development of severe muscle weakness or paralysis occurred later, was more gradual in development, and extended over a longer period.

Many mice older than 12 days, after gradually developing marked muscle weakness, recovered. In a few cases weakness was still present when the experiment was terminated after 3 weeks.

In 21–23 days old mice complete flaccid paralysis was seen in only one case, although movement of the hind legs was frequently severely impaired.

The clinical picture produced in mice inoculated with the Easton 2 strain (Type 1) agreed closely with that described for Type 1 by Beeman, Huebner and Cole (1952). In 0–1 and 3–4 days old mice in particular, death often occurred with little or no sign of paralysis, although wristdrop or paralysis of the hind limb did occur in others of the same age which had received less concentrated inocula. In older mice flaccid paralysis occurred, but did not progress as rapidly as did that produced by other strains.

The groups of mice used in these experiments were not sufficiently large in number for a critical analysis of strain differences in mean time to death in various age groups to be made. However, when estimates of mean time to death were made from the available data, although considerable differences were seen among the strains examined, it was notable that the values obtained for Easton 2 were consistently higher for all age groups at the 10 and 100 ID_{50} dosage levels than for the other five strains.

With all strains, at both dosage levels, there was a gradual increase in mean time to death with increase in age.

Weights of mice and susceptibility. After observing that in a group of 3–4 weeks old mice, small mice suffered a severe and frequently fatal infection with Dalldorf 2 while large mice were either unaffected or suffered a transient muscle weakness, the influence of weight on the susceptibility of newly weaned mice was briefly investigated.

Twenty 22–24 days old mice were divided into two groups on a weight basis. Mean weights in the two groups were 10·4 and 6·4 gm. respectively. Both groups were inoculated intraperitoneally with 0·05 ml. of a 10 p.c. suspension of 4th lab. passage virus. Details and results of the experiment are summarized in Table 2.

TABLE 2.

Influence of weight on susceptibility of weaned mice to infection with Dalldorf 2.

Group	No. of mice	Mean weight gm.	Weight range gm.	Number sick	Number dead	Mean time to death (days)
1	10	10·4	9·5–12·0	6	0	—
2	10	6·4	5·0– 8·0	10	10	6·6

The 5–8 gm. mice first showed muscle weakness on the 4th day after inoculation, and all were dead by the 10th day. Of the 9·5–12 gm. mice, 6 showed doubtful muscle weakness on the 6th day after inoculation and none died. These results indicate the necessity for keeping weight as well as age within limits as narrow as possible.

Effect of cortisone on susceptibility of newly weaned and adult mice to Dalldorf 2. Cortisone has proved effective in rendering relatively insusceptible 7–10 day suckling mice fully susceptible to infection with a group B strain (Adelaide 1). The susceptibility of weaned mice was not increased to any detectable extent.

The effect of cortisone treatment on insusceptible adult mice and newly weaned mice of variable susceptibility was therefore investigated, using the Dalldorf 2 strain.

Two groups of newly weaned mice of both sexes, of 5–7 and 9–11 gm. in weight, and one group of adult male mice of 25–30 gm. were used. These were further subdivided into three groups, receiving (1) virus only, (2) cortisone only, and (3) both cortisone and virus.

TABLE 3.

Effect of cortisone on Dalldorf 2 infections in weaned mice.

Age	Weight range gm.	Sex	Treatment	Number of mice	Number sick	Number dead	Mean time to death (days)
21–23 days	5–7	Male and Female	Virus	9	9/9	9/9	4·2
			Virus + Cortisone	9	9/9	9/9	3·0
			Cortisone controls	2	0/2	0/2	—
21–23 days	9–11	Male and Female	Virus	9	9/9	8/9	7·5
			Virus + Cortisone	9	9/9	4/9	6·5
			Cortisone controls	2	0/2	0/2	—
9–10 weeks	25–30	Male	Virus	9	0/9	0/9	—
			Virus + Cortisone	9	0/9	0/9	—
			Cortisone controls	2	0/2	0/2	—

Cortisone, 0·1 mg. per gm. body weight, was injected intraperitoneally 30 minutes before the inoculation of virus by the same route. The dose of virus was 0·1 ml. of a 10 p.c. suspension containing 500 units of penicillin and streptomycin per ml. Details and results of this experiment are recorded in Table 3.

In 25–30 gm. adult mice no signs of infection were seen in either the treated or untreated groups. In 9–11 gm. newly-weaned mice more deaths (8/9)

occurred in untreated than in treated mice (4/9) although all mice in both groups showed signs of infection. In 5–7 gm. newly-weaned mice all mice died in both groups. The groups were too small for the minor differences in mean time to death to be considered of any importance.

In all, the results indicate that cortisone, at the dosage level used, had little effect on the susceptibility of newly-weaned or adult mice to infection with the Dalldorf 2 strain. However, Sulkin, Wallis and Donaldson (1952) have shown that susceptibility to infection with this virus type may be increased by cortisone, but in their experiments the dose of cortisone employed was several times greater than that used in the above experiments.

Group B strains.

Age and susceptibility to lethal and sub-lethal infection. In addition to the two strains, Connecticut 5 and Adelaide 1, whose A.S.R.'s have been described previously, A.S.R.'s of a further three group B strains, Ohio R, Nancy, and Adelaide 2, have been determined. LD_{50} and ID_{50} titres of virus pools obtained in various age groups with each of these strains are presented in Table 4.

TABLE 4.

Age susceptibility relationships for Group B strains of "C" virus.

LD_{50} and ID_{50} titres of virus pools in mice of different age groups.

Age of Mice	Ohio R (Type 2)	Nancy (Type 3)	Adelaide 2 (Type 3) (1)	Adelaide 2 (Type 3) (2)
0–1 days	6·4††	6·9	6·6	6·8
	6·5	6·9	6·7	7·0
3–4 „	4·8	6·7	5·2	<4
	5·6	6·7	5·5	<4
7–8 „	$<3·0$	(3·0)	<1	N.D.
	5·5	4·7	<1	
9–10 „	(1·0)**	(1·7)	<1	N.D.
	3·6	6·8	<1	
12–13 „	3·4	4·3	<1	N.D.
	4·5	6·6	<1	
16–17 „	$<4·0$	5·4	1·4	<1
	4·7	6·7	$>4·0$	2·0
21–23 „	3·0	4·5	<1	N.D.
	3·2	6·2	<1	
4–5 weeks	2·2	(2·0)	<1	N.D.
	3·2	5·7	<1	
6–7 „	N.D.*	3·4	N.D.	N.D.
		4·5		
8–9 „	N.D.	4·7	N.D.	N.D.
		5·2		

†† Upper and lower values represent LD_{50} and ID_{50} titres respectively.

* N.D. = not done.

** Brackets indicate approximate titre.

With all strains investigated a pronounced fall in susceptibility occurred during the first days of life, reaching a minimum at or near the tenth day when lethal infections alone were considered, the fall in susceptibility being less marked and reaching the lowest value earlier when total infections were considered. Although this feature was common to all strains, large variations occurred between strains in the dosage levels required to produce infection or death in older suckling and weaned mice, and in the relationship between these dosage levels (Fig. 3).

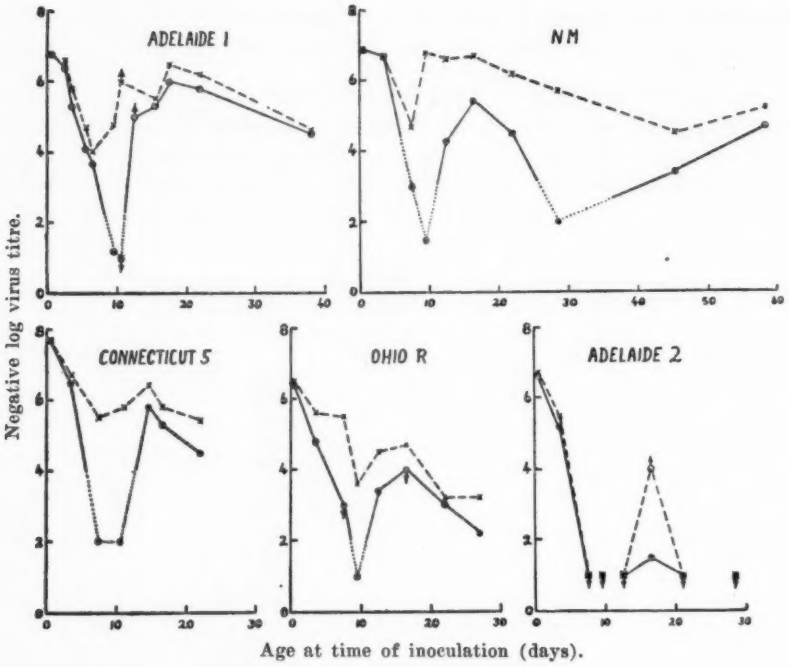

Fig. 3. Age-susceptibility relationships of Group B strains. Upper interrupted line = ID_{50}. Lower continuous line = LD_{50}. Dotted line = approximate titre. * NM = Nancy.

Thus the Nancy strain produced infections in older suckling and weaned mice at dosage levels comparable to those required for Adelaide 1 infection, although a much higher dosage was required to produce lethal infections than with the Adelaide 1 strain. With the Ohio R strain both lethal and sub-lethal infections in mice greater than 2 weeks old were more difficult to establish, although the differences between the lethal and infectious doses were smaller than for Nancy.

The Adelaide 2 strain presented a great contrast to the other group B strains in its A.S.R. Despite LD_{50} and ID_{50} titres in 0–1 day old mice of the same order as those of other group B strains, susceptibility fell rapidly to an undetectable level in 7–8 days old mice and was regained to a measurable extent in 16–17 days old mice only. No deaths, but a few infections, were detected in 3 and 4-week old mice.

Considered as a whole, these experiments show that although all the group B strains examined possess the same degree of infectivity and pathogenicity for 0–1 day old mice, these properties are lost to varying degrees with increase in age with different virus strains. The descending order in which these strains fell with respect to A.S.R.'s was roughly Adelaide 1, Nancy, Connecticut 5, Ohio R and Adelaide 2.

The nature of disease in mice. The main signs of infection with the Nancy strain in suckling mice infected during the first week of life were emaciation and weakness. Death usually occurred within two weeks of infection. Tremors and paralysis were rare in infection with this strain.

The Ohio R strain produced muscle weakness or paralysis more frequently than the Nancy strain, approximately 20 p.c. of infected mice in the 0–1 and 3–4 days age groups being affected in this way. Paralysis was most frequently seen in mice inoculated with the dilutions of virus near the titration end point. The remaining mice exhibited emaciation and weakness prior to death, or died without prior signs of infection.

In contrast to the above strains, the Adelaide 2 strain regularly produced ataxia and, to a lesser extent, wristdrop and cachexia in suckling mice less than 4 days old.

In older suckling and weaned mice the disease did not differ from one strain to another. The disease was more protracted and was marked by loss of weight, ruffled fur, and a hunched back. Death occurred from 3 days to 4 weeks after infection, occasionally without signs of disease having been seen.

The Adelaide 1 and Connecticut 5 strains in a comparable series of experiments were shown to produce the emaciation and weakness characteristic of pancreatitis, as the main feature of infection in mice of all ages.

By arranging strains in order on the basis of the type of disease produced, and therefore on the basis of tissue tropisms, the five strains examined fell in essentially the same order as that indicated by A.S.R.'s. The dependence of A.S.R.'s of virus strains on the tissue tropisms of these strains is thus indicated.

Route of inoculation and susceptibility of 16–17 days old mice to infection with Adelaide 2. The nature of the A.S.R. of this strain suggested that susceptibility of the brain falls more rapidly than that of the pancreas. The possibility remained however, that a recovery of susceptibility comparable to that of the pancreas might be masked in older mice by the constitutional barriers

which render some neurotropic viruses non-pathogenic for older mice when inoculated by peripheral routes.

However, when groups of 8 mice 16–18 days old were inoculated intracerebrally and intraperitoneally, only those inoculated intraperitoneally became sick.

DISCUSSION.

When these experiments were begun it was hoped that A.S.R.'s might prove suitable as a means of determining degrees of relationship in biological properties between serological types within the B group.

Dalldorf and Gifford (1952) have recently shown the importance of the history of a strain in determining the nature of the disease produced. In this paper, which unfortunately was not seen until the experiments described above had been completed, it was shown that Connecticut 5, after serial brain passage, was stable and produced only brain lesions. After pancreas passage, pancreatitis was marked, and after muscle passage, the effects produced were intermediate to those of brain and pancreas passage. These observations imply that the A.S.R. of a strain will also be determined by its history. They explain what was at the time a somewhat surprising observation: that the Adelaide 2 and Nancy strains, although very different in the nature of their A.S.R.'s, were shown to be closely related when examined by means of cross neutralization tests.

The virus strains used in these experiments have been maintained in this laboratory by passage of muscle-bone suspension. The Connecticut 5, Ohio R and Nancy strains were received from Dr. Melnick as carcases in glycerol saline. The one exception to the above is the Adelaide 2 strain, the second passage of which was made using a suspension of both muscle and brain. Subsequent passages have been by means of muscle-bone suspensions. The passage number of each of the group B strains used in the above experiments is indicated in Table 5.

TABLE 5.

Number of passages of Group B strains used in age-susceptibility experiments.

Strain	Number of passages in this laboratory	Total number of passages
Connecticut 5	2	9
Ohio R	1	5
Nancy	3	>3
Adelaide 1	10	10
Adelaide 2	8	8

The observations of Dalldorf and Gifford on the importance of the passage history of a group B strain in determining the type and extent of tissue tropism of a given strain in mice, renders A.S.R.'s of little value in indicating the degree of biological relationship between strains of this group where previous history has not been taken into account. It seems, however, that such relationships, together with the clinical picture of infection in mice, do indicate clearly the main tissue affinity of a particular strain at any particular stage of its history, the A.S.R.'s affording a quantitative method for comparing the tissue affinities of a strain at various stages, or for comparing different strains. Determination of the tissue affinities by this means should prove as reliable and less tedious than histological methods.

Although passage history may decide and produce great variations in the tissue affinities of one strain, it is nevertheless possible that different serological types within the B group might prove to vary in the rate at which these affinities are developed, and the extent to which such development progresses when the "adaptive" processes using various tissue lines are complete. A.S.R.'s may therefore, under suitably controlled conditions, be of value in the comparison of group B strains with a view to obtaining some idea of the biological affinities of various serological types.

Among group A strains, where once again A.S.R.'s were determined after varying numbers of passages of different strains, differences between strains were slight, the one exception being the Easton 2 strain, and it is of some interest that this strain has been shown by other workers to differ from other group A strains in the nature of the disease produced (Beeman, Huebner and Cole, 1952), and in the results of ultra-filtration and ultra-centrifugation experiments in which, although estimates of size by ultra-centrifugation of this strain have agreed with those of other group A strains, estimates by filtration have been appreciably lower (Melnick *et al.*, 1951).

Comparison of groups A and B reveals that strains whose main or only tropisms are myotropism, pancreatotropism and neurotropism produce A.S.R.'s which differ clearly from each other.

Pathogenicity of myotropic strains for mice does not fall to any great extent until at least 4 days after birth, and often not until after the 10th day. When susceptibility does decline, it does so rapidly. With neurotropic and pancreatotropic strains the initial fall in susceptibility is immediate and more rapid than that with myotropic strains, while with pancreatotropic strains only, susceptibility after the initial fall is regained, rising in some cases to a high level. Alone, or considered together with the nature of the disease produced in mice, these relationships differentiate clearly between group A and group B strains.

SUMMARY.

The relationship of age of mice to susceptibility to intraperitoneal inoculation of six group A and five group B strains of "C" virus have been determined. Results indicate that the age susceptibility relationship, together with the nature of the disease produced, is characteristic of the group to which a strain belongs.

Variations within the groups were more marked for group B than group A, and relationships of group A strains were simple in form, while those of group B were complex. These findings are discussed.

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CONGENITAL LYMPHATIC OEDEMA IN AYRSHIRE CALVES

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Congenital oedema in Ayrshire calves has been recorded in Scandinavian countries, in Scotland and in New Zealand (Hancock, 1950; Donald, Deas and Wilson, 1952). Donald *et al.* analysed data from 325 calves with oedema, paying particular attention to the genetical nature of the condition. The term "dropsical calves," as used by these authors, does not seem adequate as oedema can arise in a variety of ways. Although they were aware of this difficulty and went to considerable lengths to ensure that their material was homogeneous, their clinical and pathological description of the condition is not complete. This suggests the need for sound diagnostic criteria before a genetical analysis of congenital abnormalities is undertaken. It seems that some of the calves described by these authors are similar to the two calves with congenital oedema described herein. These are believed to be the first recorded in Australia.

Clinical Observations.

History. The calves were obtained from a grade Ayrshire herd in the Camden district, New South Wales. They were among progeny of a bull mated with 15 of his half-sisters. Both calves were oedematous at birth and the delivery of one was assisted by the owner. One animal was a heifer and the other a bull calf. When presented for investigation on 16th September, 1952, they were five weeks and ten days old respectively.

Physical examination. Both calves were alert and bright. Their disposition was normal and growth was not retarded. Their exercise tolerance was not notably deficient, although their movements were a little clumsy. Their cardinal signs were essentially normal. Their body temperatures were 102·9° F. and 102·2° F., their heart-rates and pulse-rates were 100 and 84, and their respiratory rates 26 and 18 per minute. The pulse characters were normal, the cardiac impulse was not visible but was readily palpable and the heart sounds were normal. The breath sounds were normal and there was no distension of the abdomen or detectable ascites. The visible mucous membranes were slightly pallid. The chief abnormality in both calves was a striking oedema of the head and limbs (Fig. 1). The oedema of the head was most prominent in the intermandibular space, but it also extended to involve the cheeks, the muzzle and the eyelids. On superficial examination the ears did not appear oedematous but they were slightly thickened and did pit on pressure. A small accessory lobe, approximately 2·5 cm. × 2·5 cm., arose from the dorsal surface of the left ear of the bull calf. There

was a distinct swelling of all the limbs and the tissues pitted readily on digital pressure. In each limb the swelling was diffuse and symmetrical. In the forelimbs it extended from the coronet to the elbow, and in the hind limbs, where the swelling was greatest, from the coronet to the stifle joint. There was slight oedema of the tail and the tissues overlying the sternum. In the last two situations the oedema was not readily visible but was easily detected on palpation. The younger bull calf showed a greater degree of oedema than the older heifer calf.

Diagnosis. The outstanding feature of the clinical syndrome was oedema which had been present from birth. From the point of view of diagnosis, the most intriguing feature of the condition was the distribution of the oedema. As a general rule, most conditions causing oedema lead to a swelling of the tissues in dependent parts of the body. Although the oedema in these calves was most marked at the extremities, it included such tissues as the ears and eyelids. The unusual distribution of the oedema fluid, combined with the normality of the cardinal signs, suggested that the oedema was not of cardiac, hepatic, renal, infectious, parasitic, thyroid or nutritional origin. Having excluded the more common pathological types of oedema on clinical grounds, a provisional diagnosis of congenital lymphatic oedema was made. The calves were admitted to hospital for detailed investigation. Whilst in hospital they were fed reconstituted powdered whole milk at the rate of 8 oz. daily in two feeds. This provided a protein intake of approximately 65 gm. per day. The heifer commenced to scour after 3 days and, in spite of the oral administration of streptomycin and an astringent mixture, the diarrhoea became progressively worse. The bull accepted the reconstituted diet and suffered no digestive disturbance during the 6 weeks he was kept under observation.

Fig. 1. Lateral and posterior views showing oedema of face and hind limbs.

Laboratory investigations. The results of investigations on both calves are listed in Table 1. The low serum protein levels in both calves were interpreted as being the most significant of the laboratory findings. Because of this and the more extensive nature of the oedema in the bull calf, an attempt was made to raise the serum protein level in this animal by transfusion. Citrated bovine

plasma with a total protein value of 6·7 gm. p.c., albumin 4 gm. p.c. and globulin 2·7 gm. p.c. was given intravenously, 350 ml. on 24th September and 700 ml. on 25th September. The total amount of protein given was some 70 gm. The calf's weight prior to the transfusion was 83 lb. and was unchanged in the period following the transfusion. Serum protein values before and after transfusion are shown in Table 2. No clinical change in the calf's condition followed the transfusion and the oedema showed no sign of regression.

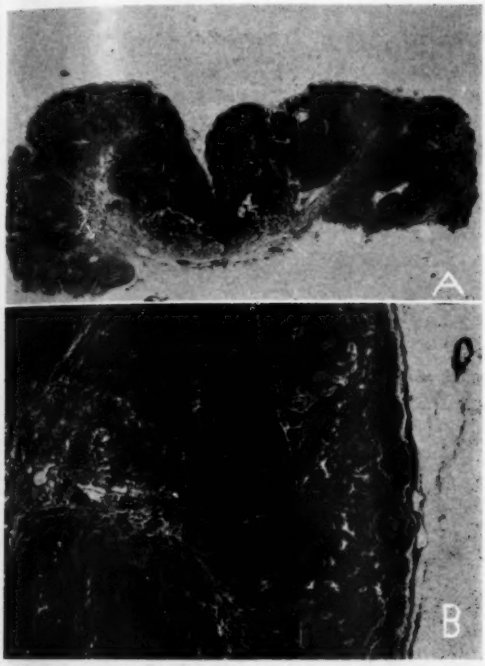

Fig. 2. Portal lymph node, heifer. (A) Very low power. Cortex relatively normal. Oedema of medulla. (B) Low power. Cortex.

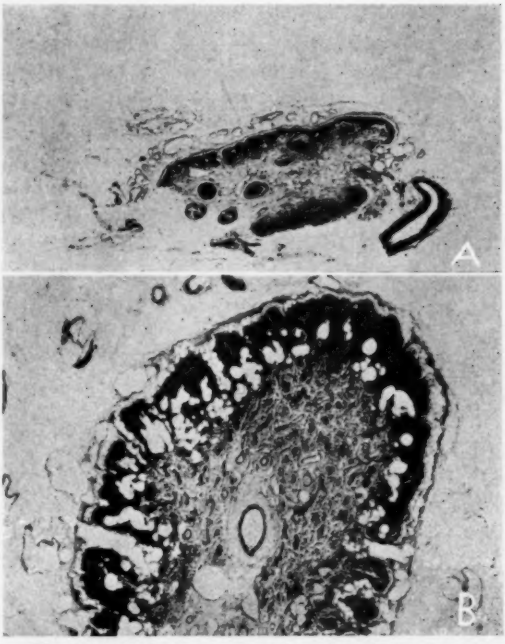

Fig. 3. (A) Left precrural lymph node, heifer. Very low power. Intranodal and extranodal lymphangiectasis and diminution of cortical tissue. (B) Right axillary lymph node, bull. Very low power. Dilatation of subcapsular sinus and of cortical lymph channels.

The electrophoretic pattern of the bull calf's serum was examined, together with the serum of a normal calf of the same age. The patterns appeared essentially similar except that that of the oedematous calf showed an almost complete absence of those proteins with the lowest electrophoretic mobility. This finding indicated that the globulin fraction was deficient and since the pattern was determined when the calf was 16 days of age, it is probable that it had not absorbed colostral globulin. It has since been learnt from the owner that the calf was unable to suckle immediately after birth, and was fed colostrum by hand.

TABLE 1.

	Heifer	Bull
Blood count:		
Hb.	10·1 gm. p.c.	10·1 gm. p.c.
Red cells	8,240,000	7,830,000
White cells	7,760	7,360
Differential:		
Neutrophiles (segmented)	55 p.c.	36 p.c.
Lymphocytes	42 p.c.	54·5 p.c.
Monocytes	3 p.c.	8·5 p.c.
Blood glucose	109 mgm. p.c.	145 mgm. p.c.
Serum protein	5·4 gm. p.c.	4·7 gm. p.c.
Serum chlorides	405 mgm. p.c.	405 mgm. p.c.
Serum sodium	462 mgm. p.c.	410 mgm. p.c.
Oedema fluid protein	1·5 gm. p.c.	1·24 gm. p.c.
Oedema fluid sodium	423 mgm. p.c.	423 mgm. p.c.*
Oedema fluid potassium	12·5 mgm. p.c.	12·5 mgm. p.c.*

* Pooled aliquots from each calf.

Urine samples from both calves were negative for sugar, protein, blood, casts and epithelial cells.

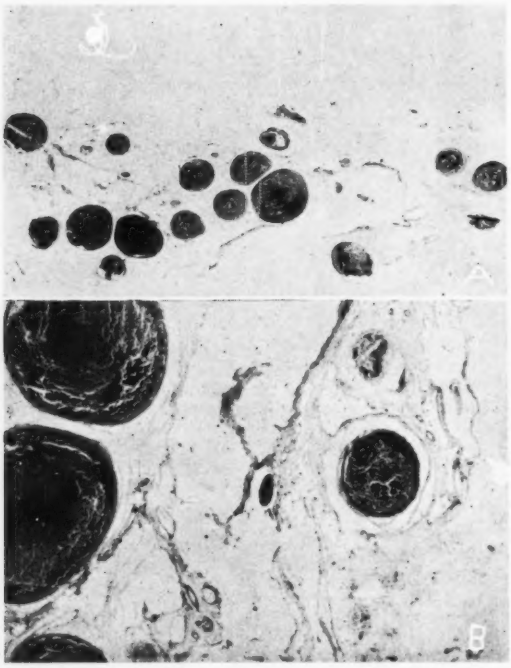

Fig. 4. Right precrural lymph node, bull. (A) Very low power. Showing isolated lymphoid follicles. (B) Low power. Showing dilated lymphatic vessels surrounding follicles.

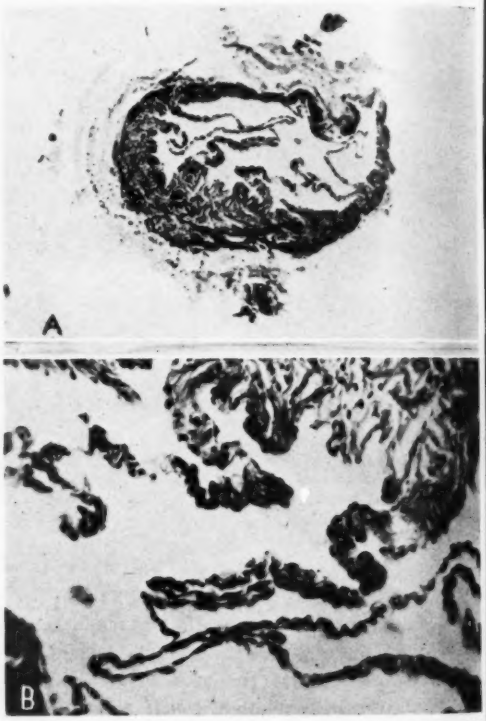

Fig. 5. Lymph vessel from right axilla, bull. (A) Low power. Intimal proliferation and strand formation. (B) High power view showing detailed structure of strands.

TABLE 2.

	Total protein gm. p.c.	Albumin gm. p.c.	Globulin gm. p.c.
Before transfusion	4·9	3·5	1·4
Immediately after	5·1	3·6	1·5
24 hours after	4·9	3·4	1·5

Further investigation of the heifer was deemed unprofitable due to the development of severe dysentery, and she was autopsied eight days after admission. The post-mortem findings in this animal pointed to an anatomical and mechanical derangement of lymphatic function, and it was considered that further attempts at treatment of the bull would be futile. Twenty-four hours before autopsy an attempt was made to assess the function of the fore and hind limb lymphatics of the bull calf. Twenty ml. of dye-albumin solution (10 mg. T1824 per ml. of a 10 p.c. solution of crystalline bovine albumin) was injected subcutaneously into one fore and one hind limb near the fetlock. Absorption of the large molecular dye-protein complex occurs only by way of the lymphatic system (Courtice and Simmonds, 1949). Serum samples taken over the following 12 hours failed to reveal any detectable dye in the circulating blood. This suggested that there was little movement of lymph from the oedematous skin even though the calf was exercised.

PATHOLOGICAL OBSERVATIONS.

Post-mortem examination.

Each calf in turn was destroyed by intravenous barbiturate administration. The general distribution of the oedema confirmed the clinical findings. The oedematous areas were gelatinous in appearance, containing much straw-coloured fluid which dripped from the cut surface. In the bull calf the dye-protein complex had not moved from the injection site in the hind limb, while in the fore limb a very small proportion had reached the prescapular lymph node via an afferent lymphatic vessel, and a trace had penetrated to one of the cranial mediastinal nodes. However, nearly all the dye had remained at the injection site in the distal region of the fore limb.

The superficial lymph nodes were variable in size and appearance; most were smaller than normal with numerous cystic dilations, more particularly in the medullary zones. The right precrural node of the bull calf resembled a mulberry. The afferent and efferent lymphatic vessels could be seen on careful dissection to be enlarged, tortuous and dilated. In the limbs the intermuscular fascial planes were much more prominent than normal, and the same appearance characterized the intramuscular connective tissue, so that the muscles appeared

pale and excessively fibrous. In the peritoneal cavity the subserous connective tissue was denser and more opaque than normal, the serosa appearing dead white. There were 50 ml. of straw-coloured fluid in the peritoneal cavity of the heifer calf, and 100 ml. of similar fluid in the peritoneal cavity of the bull calf. In the latter case a clot formed on allowing this fluid to stand. The liver in each calf was slightly small, the borders being rounded and the liver tissue friable. In the heifer calf there was a well-marked diffuse white stippling suggestive of a biliary cirrhosis. The intrahepatic bile ducts were prominent, their walls enlarged, the structures appearing as ill-defined cream-coloured streaks and spots throughout the liver substance. In this animal, too, the gall bladder was enlarged, measuring 10 × 8 × 7 cm.; the contents were normal and the duct patent. In both calves the adrenal cortices were narrow, being reduced to a rind of about 1 mm. in thickness. The adrenal cortex contained a black pigment on the left side in the heifer calf, and, to a milder degree, on both sides in the bull calf. The lymph nodes of the peritoneal cavity were normal in size for the most part, a few were slightly juicy on section. In each calf there was a mild degree of congestion of the small intestine, and in the heifer the gut contents were bloodstained.

In the thoracic cavity the pleura showed the same dead white appearance as did the peritoneum. In the heifer calf there were 100 ml. of clear amber pericardial fluid, and in the bull calf 15 ml. of a similar fluid. Simple dissection revealed that the thoracic duct of the heifer was present and dilated. In the bull calf a more complete dissection, performed with the aid of the injection of dye into the duct at post-mortem, revealed that although the duct eventually opened into the venous system at the jugular confluence, it was extremely tortuous throughout its course, and excessive pressure was necessary to propel dyed fluid along the duct. The only grossly abnormal thoracic lymph nodes were the sternal nodes of the bull calf, which were enlarged and soft in consistency.

Around the head, and more especially in the region of the intermandibular space, the skin and subcutaneous tissues were oedematous, being some 3 cm. in thickness. There was no differentiation between the skin and subcutaneous tissue in these regions, which had a dense white oedematous appearance and from the cut surface of which clear fluid dripped away. The lymph nodes in this region were small and difficult to find. In the bull calf the thyroids weighed 15 gm.

Histopathological findings.

The lymph nodes of both animals showed, in varying degrees of severity, a series of striking morphological changes. Those more centrally situated, such as the bronchial, mesenteric, internal iliac, hepatic, lumbar and deep cervical nodes, were least severely affected (Fig. 2). Some of these showed merely

diminution in lymphopoietic activity with some distension of cortex and medulla with oedema fluid, and an increase in the number of efferent lymphatics situated in the hilar region. The walls of these efferent vessels were thin, and the vessels themselves were dilated. In one of these, however, there was a greater degree of lymphangiectasis of the medullary vessels, and a fibrous thickening of their walls, the whole leading to a considerable loss of medullary lymphoid cellular tissue.

The peripheral nodes were more severely affected. The subcapsular sinusoids and afferent lymphatics were dilated (Fig. 3). In the most severely affected nodes there was, in addition, distension of the intranodal sinusoids, with the production of large, apparently multiloculated, cavities in the cortical tissue, which was reduced to a series of strands of pulp surrounding these cavities (Fig. 4). The right precrural node of the bull calf consisted merely of a number of isolated lymphoid follicles which were undifferentiated except for the presence of a subcapsular sinus (Fig. 4a). These were quite discrete, and set in a mass of loose connective tissue containing large numbers of lymphangiectatic vessels (Fig. 4b). Scattered petechiae were present in the lymphoid tissue. A proportion of the dilated vessels, both in and surrounding the affected nodes, showed striking degrees of intimal proliferation with a tendency toward the formation of strands of endothelium stretching across the lumen of the vessel, leading occasionally to multiple subdivision of the lumen (Fig. 5).

The liver of the heifer calf showed marked proliferation of bile ducts. In the portal tracts were numerous focal aggregations of cells which proved to be areas of active extramedullary haemopoiesis. These changes were not found in the liver of the bull. In the lung of the heifer there was a distension of the interlobular septa and lymphangiectasis. In the adrenals there was a varying degree of melanotic infiltration into the cortex and the capsule. The cortex was reduced in thickness and there was little lipoid present. The zona glomerulosa appeared hyperplastic, and in the medulla the more centrally situated cells showed a loss of cytoplasmic stain. Subcutaneous and subserous connective tissue showed an increase in collagen and an oedema characterized by distension of the tissue spaces. There was very little extracellular protein coagulum present in the oedematous areas. The presence of an acute enteritis was confirmed, while sections of the thyroid, central nervous system, spleen, kidney, voluntary muscle and thymus were normal.

DISCUSSION.

The pathological findings confirmed the clinical diagnosis. There was evidence of a developmental abnormality of the lymphatic system, affecting in particular the peripheral lymph nodes and their associated vessels. The important features were an atrophic condition of the lymphoid follicles, associated with

striking duplication of lymph vessels, some of which had hyperplastic walls whilst others were thin-walled and widely dilated. In most cases there was extensive proliferation of the endothelium of these vessels leading to the formation of strands of tissue stretching across the lumen of the vessel, and in many cases dividing it completely. Whilst these structures bore an obvious relationship to the valves present in normal lymphatics, their morphology was more complex and very probably provided a considerable resistance to lymph flow. In addition, the thoracic duct of the bull calf, at least, was extremely tortuous and presented an obvious resistance to the passage of fluid. These morphological abnormalities, presumably occurring during the development of the lymphatic system, would result in a decreased centripetal lymph flow with the accumulation of tissue fluid at the periphery of the lymphatic chains. As the more peripheral nodes and vessels were the most severely affected, the oedema appeared characteristically in the regions drained by these nodes.

The development of the mammalian lymphatic system has been studied extensively by many workers (von Recklinghausen, 1862; His, 1863, Huntington and McClure, 1908; Clark and Clark, 1918, 1932; Clark, 1912; and Sabin, 1908, 1911, 1916). In the cat and pig in particular, the periods at which the differentiation of the various components of the lymphatic system occur are well established.

The apparent disturbance in endothelial proliferation which both these calves showed throughout their peripheral lymphatic system is significant. His (1863), in a study of the origin of blood vessels, found they arose by differentiation of vaso-formative cells or angioblasts. These angioblastic cells differentiate out of the embryonic mesenchyme and form the endothelium, which is the characteristic tissue of the vascular system. The period during which the endothelial spindle cells differentiate from embryonic mesenchyme is considered to be limited, and Stockard (1915) showed this stage of differentiation to occur in the living chick by the end of the second day of incubation when the circulation is established. This limited period of endothelial differentiation is followed by the much more protracted period of growth. The lymphatic system, of which endothelium is the characteristic tissue, begins to form well after the period of differentiation of endothelium from mesenchyme has ceased. Consequently, congenital abnormalities in lymphatic endothelial development are associated with disturbances of tissue growth and not differentiation.

Following the formation of the early foetal arterial and venous systems, the primary lymphatic anlagen form near the veins from whose endothelium they originate. The primary lymph sacs bud off from the anterior cardinal veins and the veins of the Wolffian body in the form of two sets of paired jugular and iliac sacs, and two unpaired sacs, the retro-peritoneal and the cisterna chyli (Sabin, 1916). From these primary anlagen, the endothelium grows out by

sprouts to form eventually the peripheral lymphatic system. The central lymphatic system thus forms first and the subsequent outgrowth of lymphatic endothelium eventually completes the peripheral system. It is from these lymphatic anlagen that the primary lymph nodes develop. These, in the pig at least, include the caudal superficial cervical and deep cervical nodes (primary nodes originating from the jugular sacs), and the deep inguinal, internal iliac, and lumbar nodes from the iliac sacs. Significantly these primary nodes in the two calves with congenital oedema showed the least pathological changes.

The secondary and tertiary nodes, which form at a later date along with the peripheral lymphatic system, were the ones which showed the most marked changes. The secondary nodes arising from the jugular sac endothelium are the nodes of the face and head. These showed marked abnormalities in their lymphoid tissue component, and particularly in the endothelial elements both within and around the nodes. The lymphatic vessels, which are developed secondarily from the jugular lymph sacs and cervical plexus, supply the ears, forehead, face, lower jaw and tongue, the superficial regions of the skin of the neck, the foreleg and the superficial lymphatics of the thorax and lungs. These were areas of characteristic oedema in the two calves.

The secondary and tertiary nodes arising from the iliac sacs are those superficially placed in the hind limb region, the external iliacs, superficial inguinal, precrural and popliteal nodes. These nodes and the superficial lymphatics associated with them were grossly abnormal, and the hind limbs, flank and tail drained by these lymphatics showed extensive oedema. In the regions drained by the primary nodes, the peritoneal and pleural cavities and gut, only small amounts of oedema fluid had accumulated.

The protein content of the oedema fluid was high, and this would be anticipated from the elephantiac nature of the condition. The fact that the lymphatic system did not absorb any of the injected dye-albumin complex from the extremities indicates that the lymphatic lesions were sufficient to prevent the flow of lymph even though muscular movement (the force responsible for lymph flow in the normal animal) continued for 24 hours after injection.

It appears from this evidence that the essential nature of the congenital defect is one involving the lymphatic endothelium, at a period following the differentiation of angioblastic tissue from embryonic mesenchyme and the formation of the primary lymph sacs and nodes. The pathological changes of the peripheral lymphatic vessels, secondary and tertiary lymph nodes, indicate a disturbance in the organization and growth of lymphatic endothelium at the period during which the peripheral lymphatic system was developing. Secondary effects of pressure within and around the peripheral nodes and lymphatics no doubt contributed to the pathological changes noted.

SUMMARY.

The clinical, biochemical and pathological findings in two cases of congenital lymphatic oedema in Ayrshire calves have been described.

Clinically, the outstanding feature was oedema of the head and limbs. Biochemically, there was hypoproteinaemia and a high protein content of the oedema fluid. Pathologically, there was a developmental abnormality of the lymphatic system which was more severe in the peripheral lymph nodes and their associated vessels.

The pathological findings suggest that the congenital defect was associated with disturbances of tissue growth and not differentiation. Since the main pathological changes were found in the peripheral lymphatic vessels and the secondary and tertiary lymph nodes, it would appear that the developmental abnormality commenced at a period following the formation of the primary lymph nodes.

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BODY WATER AND ELECTROLYTE CHANGES IN BILATERALLY NEPHRECTOMIZED SHEEP[1]

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In a previous publication (Harris, McDonald, Munro and Williams, 1952) changes were described in the extracellular electrolyte pattern, blood and extracellular fluid volumes, and muscle composition of sheep, following bilateral ureteric ligation.

It was noted that the extracellular fluid volume as measured by the diffusion space of inulin fell progressively. A possibility existed that the fall in inulin space did not necessarily reflect a fall in extracellular volume, because inulin may have been concentrated in the renal pelves (i.e., outside the extracellular compartment) when it was injected immediately after ureteric ligation. This would have given a falsely high value for the initial extracellular volume determination. In fact, these initial volumes were in the range of 25–30 p.c. of body weight, whereas the average value for normal sheep is 16–18 p.c. of body weight. Partly for this reason, the investigation was repeated, anuria being produced by bilateral nephrectomy, instead of by ureteric ligation. By this change in technique the possibility of inulin being concentrated outside the extracellular compartment in this way was avoided. Another reason for repeating the investigation with nephrectomized animals was because of the claims by Mendoza, Diaz and Linazasoro (1950) and Diaz and Mendoza (1952) that the biochemical changes and clinical course of nephrectomized dogs differed from those of animals with ligated ureters. These authors claimed that the presence of renal tissue in the body considerably modified the effects of anuria. Thus it was of interest to see whether similar considerations applied to the sheep.

EXPERIMENTAL METHODS.

Cross-bred wethers were used, weighing between 24 and 40 kg. The animals were anaesthetized with "Nembutal" administered intravenously (30 mg./kg. body weight), and the kidneys were removed through loin incisions. Both kidneys were removed at the one operation in two animals, and in the other four the second kidney was removed one week after the first.

Blood for analysis was taken daily, and inulin, thiocyanate and antipyrine space determinations were made at intervals of 2–3 days.

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Chemical Methods.

Details of the methods used for collection of blood, and the determination of blood pH, haematocrit, plasma Na, K, HCO_3, Cl, HPO_4, SO_4, and protein have been given in a previous publication (Harris, McDonald, Munro and Williams, 1952).

Inulin space. Inulin space was determined by injecting a measured volume of 10 p.c. inulin from a calibrated syringe, and taking the plasma water concentration one hour later as representing the concentration of inulin in the extracellular fluid at equilibrium. This method was used in the preceding paper (Harris *et al.*, 1952). The validy of this procedure is discussed later.

Thiocyanate space. Thiocyanate space was determined by the method of Crandall and Anderson, as described by Consolazio, Johnson and Marek (1951). The plasma water concentration of thiocyanate one hour after injection was used in calculating the diffusion space.

Antipyrine space. Antipyrine space was determined by the method of Soberman *et al.* (1949). Three or four plasma samples were taken at 1-hour intervals following intravenous injection of 2 gm. of antipyrine. The decay curve of antipyrine concentration plotted logarithmically and calculated by the method of least squares, was extrapolated to zero time.

TABLE 1.

Antipyrine, inulin and thiocyanate space.

	Day	0		2		4		7	
		l	p.c.	*l*	p.c.	*l*	p.c.	*l*	p.c.
568	Antipyrine	16·8	(53)	16·3	(51)				
	Inulin	5·9	(18)	4·5	(14)				
569	Antipyrine	13·2	(54)	15·4	(67)				
	Inulin	5·7	(23)	5·7	(24)				
1036	Antipyrine	15·7	(47)	12·8	(39)			15·0	(49)
	Inulin	7·0	(21)	8·0	(25)			4·4	(14)
	Thiocyanate	6·8	(20)					11·5	(37)
1037	Antipyrine	26·2	(65)	15·8	(42)			18·5	(53)
	Inulin	6·3	(16)	5·6	(15)			3·4	(10)
	Thiocyanate	10·8	(27)	23·6	(63)	45·0	(136)	10·3	(29)
1015	Antipyrine	16·2	(55)			17·3	(62)	21·3	(78)
	Inulin	5·9	(21)	4·4	(15)	4·2	(15)	4·4	(16)
	Thiocyanate	14·8	(50)	14·2	(49)	11·1	(39)	10·0	(37)

CLINICAL COURSE AND SURVIVAL TIME AFTER REMOVAL OF SECOND KIDNEY.

As with the previous series (Harris, McDonald, Munro and Williams, 1952), post-operative food intake was negligible and water intake was variable. All animals lost weight following bilateral nephrectomy (Fig. 1). The only variation occurred during the first post-operative day in animals 1015 and 1038. These two animals gained 1·9 and 0·25 kg. respectively on this day.

The two animals (568 and 569) whose kidneys were both removed at the one operation survived less than four days, whereas those subject to the two-

stage operation survived from 6 to 10 days. (The animal which survived 10 days, 1015, was moribund on the morning of the 9th day and recovered temporarily following one 3-hour haemodialysis, using an Alwall artificial kidney). Thus, although the numbers are too small for statistical significance, it would appear that the severity of surgical trauma may be a factor determining post-nephrectomy survival time.

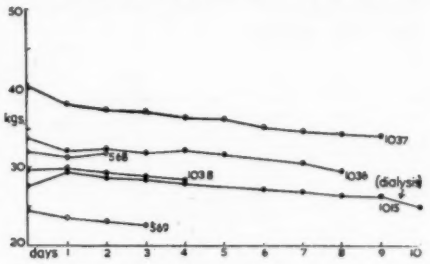

Fig. 1. Body weight changes following bilateral nephrectomy. Both kidneys were removed at the one operation in 568 and 569.

Diarrhoea developed in five of the six sheep and occurred on the day of death in four of these (569, 1037, 1038 and 1015). Sheep 1036 developed diarrhoea on the 4th day, but there was no evidence of diarrhoea on the 6th, 7th and 8th days.

PLASMA ELECTROLYTE CHANGES (*Tables 2–7*).

Blood pH. The blood pH rose on the first post-operative day in two sheep (1038 and 569) and no significant change was observed on the first post-operative day in three (568, 1036 and 1037). After the first day, the blood pH fell until death, except in the case of 568. Blood pH was not measured in the case of 1015. In three sheep, 569, 1036 and 1037, the fall in pH was much greater than occurred in the previous series, final readings being 7·18, 6·90 and 7·00 respectively. No significant change occurred in 568.

TABLE 2.

Bilateral nephrectomy. One stage.

Sheep No. 568.

Day	0	1	2
Blood pH	7·26	7·29	7·28
Plasma Na m.eq./l.	147	144	134
„ K „	4·6	5·4	5·4
„ HCO_3 „	24	28	19
„ Cl „	107	103	96
„ SO_4 „			2·37
„ HPO_4 „	1·51	1·03	1·37

Plasma sodium. The plasma sodium concentration fell in all animals from a pre-operative average of 147·5 to an average of 138·0 m.eq./l. on the day before death. The rate of fall varied from animal to animal. In 1038 an early fall occurred, and the lower concentration was maintained. In 1036, 568 and 569

the fall was progressive, although in the case of 1036 Na concentration rose from 133 to 140 m.eq./l. on the day of death. In 1037 and 1015 the Na concentration fluctuated within the range of ± 2 m.eq./l. for the first five days, and then fell rapidly.

TABLE 3.

Bilateral nephrectomy. One stage.

Sheep No. 569.

Day	1	2	3
Blood pH	7·37	7·45	7·18
Plasma Na m.eq./l.	142	141	135
„ K „	4·6	6·6	6·7
„ HCO_3 „	22	21	16
„ Cl „	101	101	97
„ SO_4 „	0·7	4·8	5·6
„ HPO_4 „	0·81	0·59	0·57

Plasma chloride. The plasma chloride concentration fell in all animals from a pre-operative average of 105 m.eq./l. to an average of 93 m.eq./l. on the day before death. This fall was progressive in 568 and 1038, but in 1015 and 1037 the Cl concentration fluctuated in the normal range for the first 4–5 days, after which a fall occurred. In the case of 1036, the Cl concentration fell progressively in the first three days, then rose to normal on the next day, and fell progressively again. In the case of 569, the concentration fell on the first day and remained the same the following day.

In 1036, 1037 and 1038 the ratio Cl : Na fell from an average pre-operative value of 0·70 to an average of 0·64 on the day before death. In the other three the Cl : Na ratio remained either constant or rose slightly.

TABLE 4.

Bilateral nephrectomy. Two stage.

Sheep No. 1015.

Day	0	1	2	3	4	5	6	7	8	9	(9)*
Hct. p.c. cells		42	52	41	37	41		39		34	20
Plasma Na m.eq./l	147	149	148	145	145	147	145	140	141	139	141
„ K „	3·4	3·5	6·0	5·6	5·7	6·1	6·4	6·2	6·8	6·8	6·2
„ Mg „	2·4	1·1	3·8	3·8	3·8	4·0	4·5	4·2	4·6	4·4	4·1
„ HCO_3 „	23	24	23	22	19	21	21	16	18	16	17
„ Cl „	110	111	113	110	109	109	102	100	97	97	95
„ HPO_4 „	1·0	1·3	1·1	1·4	1·1	1·0	1·5	2·0	2·6	3·1	2·4
„ SO_4 „	0	0	2·3	0·9	1·2	2·1	2·9	4·7	5·0	10·1	3·1
„ Protein „	18	19	18	18	18	17	19	19	19	18	20

* Following haemodialysis with Alwall artificial kidney.

Plasma bicarbonate. The plasma bicarbonate concentration fell in all animals, the fall being greatest in 1036 and 1037, whose final concentrations were 6 and 10 m.eq./l. respectively. In three animals (568, 1036 and 1037) a slight rise occurred on the first post-operative day.

TABLE 5.

Bilateral nephrectomy. Two stage.

Sheep No. 1036.

Day	0	1	2	3	4	5	7	8	9
Het. p.c. cells	49	45	36		37		39	41	
Blood pH	7·34	7·36	7·28	7·26	7·19	7·20	7·10	7·10	6·90
Plasma Na m.eq./l.	145	140	137	140	133	135	133	140	
„ K „	3·9	4·8	4·8	6·0	6·5	6·5	7·5	8·9	
„ HCO_3 „	20	22	16	16	15	10	8	9	6
„ Cl „	104	108	100	94	104	100	101	98	97
„ HPO_4 „	1·2	0·8	1·2	1·3	1·4	1·7	1·8	1·9	
„ SO_4 „	1·0	1·5	2·1	1·5	4·1	8·7	8·2	9·7	
„ Protein „	18	18	18	18	20	18	21	22	

Plasma potassium. In three animals (1015, 1036 and 1037) a progressive rise occurred of 3·3, 5·0 and 2·2 m.eq./l. respectively. A small post-operative rise occurred in 568, 569 and 1038, and this new level was maintained until death.

Plasma sulphate and phosphate. The plasma sulphate concentration rose progressively to abnormally high levels in all animals except 568, in which the only sulphate estimation, on the day before death, was slightly above normal. Very little change occurred in the phosphate concentration. These findings are similar to those in the previous series.

Plasma protein. There was a slight rise in the plasma protein concentration in 1037, 1036 and 1015. No change occurred in 1038, and protein estimations were not done in 568 and 569.

TABLE 6.

Bilateral nephrectomy. Two stage.

Sheep No. 1037.

Day	0	1	2	3	4	5	6	7	8
Het. p.c. cells	45	36	32	27	25	20	32	24	31
Blood pH	7·39	7·33	7·30	7·25	7·18	7·12	7·11	7·10	7·00
Plasma Na m.eq./l.	154	154	152	152	154	156	150	147	140
„ K „	4·0	4·5	4·4	4·8	4·6	5·3	4·8	6·8	6·2
„ HCO_3 „	19	22	16	16	15	15	12	11	10
„ Cl „	106	107	114	103	106	103	109	97	85
„ HPO_4 „	1·5	1·5	1·6	2·5	1·9	2·1	3·5	3·3	3·6
„ SO_4 „	0·7	1·6	2·6	3·0	2·8	6·0	6·2	6·4	7·7
„ Protein „	18	17	21	20	21	21	21	21	21

Haematocrit. The haematocrit fell in an irregular fashion in 1038, 1037, 1036 and 1015. The falls recorded were between 15 p.c. and 50 p.c. of the pre-operative reading. There was no relationship between the plasma protein changes and haematocrit, except that in a general way the direction of changes was opposite.

In general, these blood and plasma changes were similar to those of the animals with ligated ureters (Harris *et al.*, 1952), although there was a somewhat greater individual variation and an accentuation of the changes in pH and bicarbonate.

TABLE 7.

Bilateral nephrectomy. Two stage.

Sheep No. 1038.

Day	0	1	2	3	4
Hct. p.c. cells	39		37		34
Blood pH	7·28	7·35	7·29		7·23
Plasma Na m.eq./l.	150	144	142	142	142
„ K „	3·6	4·6	4·4	4·7	4·0
„ HCO_3 „	24	23	22	22	
„ Cl „	103	96	94	92	89
„ HPO_4 „	2·4	2·3	2·0	1·9	2·5
„ SO_4 „	3·2		3·8	4·3	5·0
„ Protein „	16	16			16

Inulin space. With the exception of sheep 569, the inulin space, derived in the same way as in the previous series (Harris *et al.*, 1952), fell, both absolutely, and as a percentage of body weight (Table 1). In the case of 569 there was no change on the second post-operative day, and no other estimation was done before death. In the case of 1036 the inulin space rose absolutely relatively to body weight on the second post-operative day, but had fallen to below the pre-operative level by the seventh day. With the exception of 1038, none of the animals developed diarrhoea before the last inulin space determination.

The validity of these findings as indices of change in extracellular fluid volume is, however, open to question. In these animals the plasma concentration of inulin was studied at successive intervals after injection, and it became at once apparent that equilibration of the injected inulin did not occur. The one possible exception was 568, in which stable levels of inulin occurred following injection. In the other animals the plasma inulin concentration fell in a variable manner from the peak concentration which occurred shortly after injection. The shape of the concentration curve varied from animal to animal, and even from one estimation to another on the same animal (Figs. 2, 4, 5). In some estimations this highest plasma concentration occurred at the end of the first hour, and in others it rose still further at the end of the second hour. Also, once the peak was reached, the plasma inulin concentration fell progressively in most instances and no stable level was reached. It may be significant that in the case of the two sheep whose kidneys were both removed at the one operation, the plasma inulin concentration following injection was the most stable, but interpretation of this finding, for example in terms of cell permeability, can only be of a highly speculative nature. A disturbing feature of the findings is that the concentration at the peak of the inulin concentration curve gives the most acceptable value

for initial extracellular volume (16–23 p.c. body weight) in relation to the volume obtained in normal sheep using the constant infusion technique (16–18 p.c. body weight). This peak concentration can hardly be regarded as representing an equilibrium concentration. Thus the falling inulin concentrations and the high diffusion space of inulin at times other than those corresponding to the peak, lead to the conclusion that in the majority of these animals inulin disappeared from the extracellular compartment. It cannot be decided from the available data whether this disappearance was due to diffusion of inulin into cells or to destruction of inulin. The authors feel that the former interpretation is probably the correct one, in view of the findings with injected thiocyanate (Fig. 3) and findings on muscle analysis in anuric rats (Coats, McDonald and Munro, 1953).

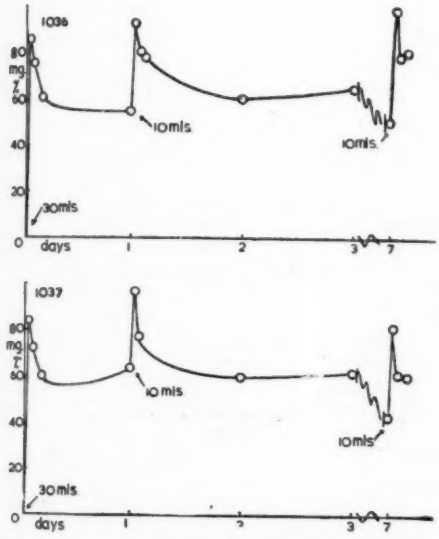

Fig. 2. Changes in plasma inulin concentration following injection of a 10 p.c. inulin solution at the points indicated by the arrows.

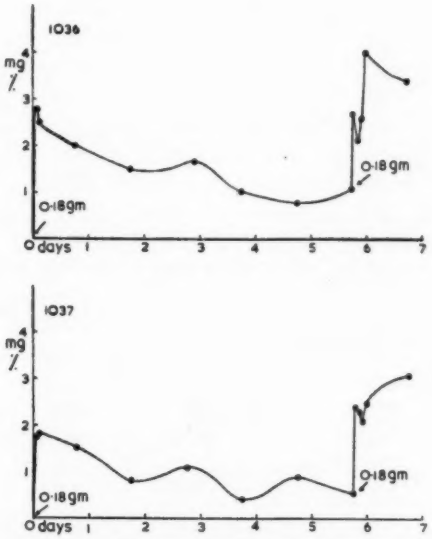

Fig. 3. Changes in the concentration of thiocyanate following the injection of an approximately 2 p.c. solution of Ca CNS.

When the two experimental series are compared, it is seen that the initial inulin diffusion space of the nephrectomized animals falls within a lower range than that of the animals subjected to ureteral ligation. Although this may be interpreted as evidence for the excretion of inulin into the renal pelvis in the case of ureteral ligation, the fact that it has now been shown that no equilibrium level was reached in nephrectomized animals makes such speculation of little value.

Thiocyanate space. Thiocyanate space was estimated concurrently with the inulin space determinations, and the findings (Table 1, Fig. 3) indicate that the same considerations with regard to the reliability of this method as an index of extracellular fluid volume in anuric animals apply. There was a wide variation between the extracellular volumes as measured by the two methods, and the thiocyanate concentration fell after injection to such an extent that the apparent diffusion space became impossibly high in some instances. In one instance (1037, Table 1) the apparent diffusion space of thiocyanate was greater than the antipyrine space on the same day, and in another the concentration fell so low that the apparent space was more than 100 p.c. of body weight. Thus the thiocyanate ion must have been actually concentrated outside the extracellular compartment, presumably within the cells.

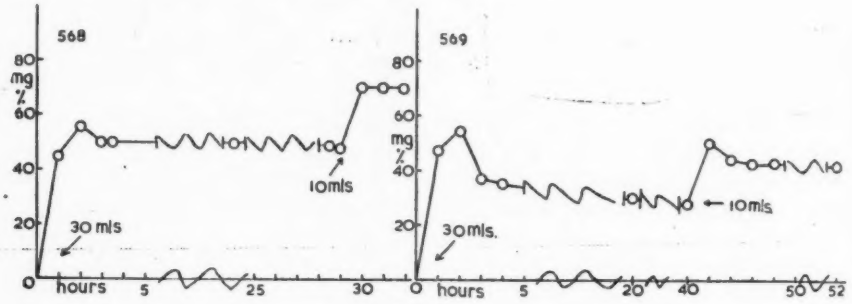

Fig. 4.

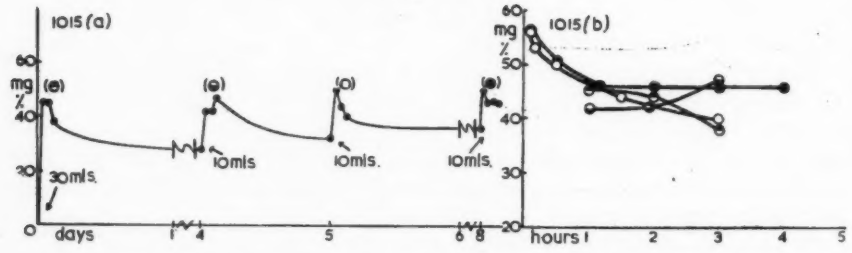

Fig. 5.

Figs. 4 and 5. Changes in plasma inulin concentration following injection of a 10 p.c. inulin solution at the points indicated by the arrows. In 1015(a), the symbols in brackets refer to the symbols used in 1015(b), where the changes are plotted on different time and concentration axes.

Antipyrine space. The reliability of this method as an index of total body water in the sheep is still being investigated. Preliminary work gives a range of 50–60 p.c. of shorn body weight in normal animals, with a rough correlation between fat deposition and differences in antipyrine space. The range is appre-

ciably higher in very young lambs. The reproducibility of the total body water determination by this method in sheep is about ± 10 p.c.

It is interesting to note that the antipyrine space rose as a percentage of body weight in the animals which survived more than 4 days, but that in two of these animals (1036 and 1037) it fell relatively to body weight on the second post-operative day. The interpretation which is put on the terminal relative rise in antipyrine space is that the body's "cell solids," especially fat, decreased and were replaced by some of the water produced in their metabolism.

DISCUSSION.

The main feature of these experiments is the evidence that inulin and thiocyanate are not confined to the extracellular compartment in anuric sheep. Similar conclusions have been reached with regard to inulin as an index of extracellular volume in anuric human beings and dogs (Finkenstaedt, O'Meara and Merrill, 1953). This is of some importance, as the conclusions reached by other authors, notably Hamburger (1951), concerning changes in the extracellular fluid volume during anuria in man and the dog, are not necessarily valid since thiocyanate or mannitol space were used as indices of extracellular volume. The difficulty in determining what happens to the volume of the extracellular fluid during anuria renders it impossible to interpret fully the changes in the extracellular ionic pattern. The falling Cl and Na concentrations may be due in part to an expansion of extracellular fluid volume or to a shift of Na and Cl into the intracellular compartment. The present authors regard the changes in anuria as being due to a diminution of extracellular volume with an increase of cell water content and penetration of sodium and chloride into cells. Evidence for this is contained in the muscle analyses published previously (Harris *et al.*, 1952) and in muscle and brain analyses of anuric rats (Coats, McDonald and Munro, in preparation). Furthermore, the earlier observation that the plasma volume of anuric sheep falls (Harris *et al.*, 1952), is more in accord with a fall in extracellular volume than a rise.

The other features of the experimental results are mainly confirmatory of the earlier observations (Harris *et al.*, 1952). It is possible that the greater surgical trauma of bilateral nephrectomy was in some way responsible for the accentuation of the extracellular electrolyte changes relative to those of the ureter-ligated animals. It is interesting that both sheep which were subjected to a one-stage bilateral nephrectomy survived only a few days, whereas the other animals survived a period of time comparable with the ureter-ligated series.

SUMMARY.

Six sheep were subjected to bilateral nephrectomy, two animals in one stage, and four animals in two stages.

The changes in plasma ionic pattern were similar to those in sheep with ligated ureters, although the fall in pH and bicarbonate concentration was more marked in the nephrectomized animals.

Attempts to study changes in body water compartments by the use of antipyrine, inulin, and thiocyanate revealed that inulin and thiocyanate are not confined to the extracellular compartment in anuric sheep. Changes in antipyrine space were consistent with a replacement of body fat or protein with water.

The animals whose kidneys were both removed at the one operation survived less than half the period of the others.

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